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(54) Title: ADZYMES AND USES THEREOF

(57) Abstract: Disclosed is a family of novel protein constructs, useful as drugs and for other purposes, termed "adzymes," comprising an address moiety and a catalytic domain. In some types of disclosed adzymes, the address binds with a binding site on or in functional proximity to a targeted biomolecule, *e.g.*, an extracellular targeted biomolecule, and is disposed adjacent the catalytic domain so that its affinity serves to confer anew specificity to the catalytic domain by increasing the effective local concentration of the target in the vicinity of the catalytic domain. The present invention also provides pharmaceutical compositions comprising these adzymes, methods of making adzymes, DNA's encoding adzymes or parts thereof, and methods of using adzymes, such as for treating human subjects suffering from a disease, such as a disease associated with a soluble or membrane bound molecule, *e.g.*, an allergic or inflammatory disease.

ADZYMES AND USES THEREOF

Related Applications

This application claims priority to U.S. Provisional Patent Application Serial No. 60/406,517, filed August 27, 2002, U.S. Provisional Patent Application Serial No. 60/423,754, filed November 5, 2002, and U.S. Provisional Patent Application Serial No. 60/430,001, filed November 27, 2002, the entire contents of each of which are incorporated herein by reference.

Background of the Invention

This invention relates to synthetic protein constructs useful in modulating a variety of targeted molecules *in situ*. In particular aspects, it relates to a family of constructs employing linked molecular parts which target and modulate the activity of a biomolecule catalytically to induce a therapeutic effect.

Many diseases are caused by or associated with biomolecules, either free in solution in body fluids or exposed to extracellular body fluids such as membrane-bound proteins and polysaccharides, such as cytokines or growth factors, and it is widely recognized that it is possible to develop therapies for such diseases by modulating the activity of the biomolecule.

For example, overproduction of TNF-α and/or TNF-β is closely linked to the development of many diseases, including septic shock, adult respiratory distress syndrome, rheumatoid arthritis, selective autoimmune disorders, graft-host disease following bone marrow transplantation and cachexia. Other diseases associated with excessive TNF-α and/or TNF-β production include hemorrhagic shock, asthma and post-renal dialysis syndrome. The multiplicity of actions of TNF-α and TNF-β can be ascribed to the fact that TNF-α and/or TNF-β actions result in activation of multiple signal transduction pathways, kinases, transcription factors, as well as an unusually large array of cellular genes. (Walajtys-Rode, Elizbieta, Kosmos (Warsaw), 44, 451-464, 1995, C.A. 124:199735a, 1995). TNFα has also been linked to the development of autoimmune disorders.

Current therapies for combating the foregoing disorders include the administration of a binding agent, such as an antibody or soluble receptor, that binds to and thereby inhibits a targeted biomolecule that causes or is associated with the disease. However, there are many drawbacks associated with this approach. For example, binding agents, by their very nature, can only inhibit the biomolecules(s) to which they are bound, and can neither catalytically inactivate a series of biomolecules nor chemically alter the bound biomolecules(s). It is probably for these reasons that relatively large doses of binding

agents are often needed to achieve therapeutic effectiveness, exposing the subject to dangerous and often toxic side-effects. Moreover, production of such large quantities of antibodies and other binding agents is expensive.

Targeted therapeutic agents with greater effectiveness than traditional binding agent therapeutics would be a desirable improvement.

Summary of the Invention

In certain aspects, the invention provides a new class of engineered protein constructs, referred to herein as "adzymes", as well as methods and compositions related to the use and production of adzymes. Adzymes are chimeric protein constructs that join one or more catalytic domains with one or more targeting moieties (or "addresses"). A catalytic domain of an adzyme has an enzymatically active site that catalyzes a reaction converting a pre-selected substrate (the "target" or "targeted substrate") into one or more products, such as by cleavage, chemical modifications (transformations) or isomerization. Such products may have an altered activity relative to the substrate, optionally having an increased or decreased activity or an activity that is qualitatively different.

In certain aspects, the invention provides adzymes comprising a catalytic domain and a targeting moiety, wherein the catalytic domain catalyzes a chemical reaction converting a substrate into one or more products, and wherein the targeting moiety reversibly binds to an address site that is either on the substrate or in functional proximity with the substrate. Preferably, the targeting moiety binds reversibly to the address site. Optionally, said targeting moiety and said catalytic domain are heterologous with respect to each other. Generally, said targeting moiety, when provided separately, binds to the substrate, and said catalytic domain, when provided separately, catalyzes the chemical reaction converting said substrate to one or more products.

In certain embodiments, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly or $SerGly_4$. In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The

linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

In certain embodiments, the adzyme is an immunoglobulin fusion, wherein the catalytic domain and the targeting moiety are joined, in a geometry consistent with effectiveness against substrate, to at least a portion of an immunoglobulin comprising a constant domain of an immunoglobulin. For example, the adzyme may comprise a first fusion protein and a second fusion protein, wherein the first fusion protein comprises a constant portion of an immunoglobulin heavy chain and a catalytic domain, and wherein the second fusion protein comprises a constant portion of an immunoglobulin heavy chain and a targeting domain that reversibly binds with an address site on or in functional proximity to the substrate. Preferably the immunoglobulin portions are Fc portions that dimerize by disulfide bonds.

In certain embodiments, an adzyme is designed so as to have one or more desirable properties, with respect to the reaction with said substrate. In many instances, such properties will be significant for achieving the desired effect of the adzyme on the substrate. For example, an adzyme may have a potency at least 2 times greater than the potency of catalytic domain or the targeting moiety alone, and preferably at least 3, 5, 10, 20 or more times greater than the potency of the catalytic domain or targeting moiety alone. An adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. An adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. An adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. An adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-3} sec^{-1}, 10^{-2} sec^{-1}, or greater. An adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. An adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the K_m of the catalytic domain alone. An adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, an adzyme will be designed so as to combine two or more of the above described properties.

A catalytic domain may include essentially any enzymatic domain that achieves the desired effect on a selected substrate. The catalytic domain may be selected so as to modify one or more pendant groups of said substrate. The substrate may include a chiral atom, and said catalytic domain may alter the ratio of stereoisomers. The catalytic domain may alter the level of post-translational modification of the polypeptide substrate, such as

a glycosylation, phosphorylation, sulfation, fatty acid modification, alkylation, prenylation or acylation. Examples of enzymatic domains that may be selected include: a protease, an esterase, an amidase, a lactamase, a cellulase, an oxidase, an oxidoreductase, a reductase, a transferase, a hydrolase, an isomerase, a ligase, a lipase, a phospholipase, a phosphatase, a kinase, a sulfatase, a lysozyme, a glycosidase, a nuclease, an aldolase, a ketolase, a lyase, a cyclase, a reverse transcriptase, a hyaluronidase, an amylase, a cerebrosidase and a chitinase. Regardless of the type of catalytic domain, it may be desirable that the adzyme be resistant to autocatalysis (e.g., inter- or intra-molecular reactions), particularly at an adzyme concentration that is about equal to the concentration of adzyme in a solution to be administered to a subject. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate.

In certain preferred embodiments, the catalytic domain of an adzyme includes a protease domain that, when active, cleaves at least one peptide bond of a polypeptide substrate. In general it will be desirable to design the adzyme such that it is resistant to cleavage by the protease catalytic domain. The protease domain may be generated as a zymogen (an inactive form) and then activated prior to use. The adzyme may be purified from a cell culture in the presence of a reversible protease inhibitor, and such inhibitor may be included in any subsequent processing or storage activities.

A targeting moiety may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting moiety will comprise a polypeptide or polypeptide complex, and particularly an antibody or polypeptide(s) including an antigen binding site of an antibody. For example, a targeting moiety may include a monoclonal antibody, an Fab and F(ab)2, an scFv, a heavy chain variable region and a light chain variable region. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In certain embodiments, the targeting moiety is a polyanionic or polycationic binding agent. Optionally, the targeting moiety is an oligonucleotide, a polysaccharide or a lectin. In certain embodiments, the substrate is a receptor, and the targeting moiety includes a ligand (or binding portion thereof) that binds to the receptor. In certain embodiments, the substrate is a ligand of a receptor, and the targeting moiety includes a ligand binding portion of the receptor, particularly a soluble ligand binding portion.

An adzyme may be used to target essentially any amenable substrate in a variety of technological applications, including therapeutic uses, industrial uses, environmental uses and uses in microfabrications. In a preferred embodiment, an adzyme substrate is from a mammal, such as a rodent, a non-human primate or a human. In a preferred embodiment,

the substrate is endogenous to a human patient. In certain embodiments, the substrate is a biomolecule produced by a cell, such as a polypeptide, a polysaccharide, a nucleic acid, a lipid, or a small molecule. In certain embodiments, the substrate is a diffusible extracellular molecule, and preferably an extracellular signaling molecule that may act on an extracellular or intracellular receptor to triggers receptor-mediated cellular signaling. Optionally, the extracellular signaling molecule is an extracellular polypeptide signaling molecule, such as an inflammatory cytokine. In a preferred embodiment, the substrate is an interleukin-1 (e.g., IL-1α, IL-1β) or TNF-α. In certain embodiments, the substrate is a polypeptide hormone, a growth factor and/or a cytokine, especially an inflammatory cytokine. Optionally, the adzyme acts to reduce a pro-inflammatory activity of a substrate. A substrate may be selected from among the following: four-helix bundle factors, EGF-like factors, insulin-like factors, β-trefoil factors and cysteine knot factors. In certain embodiments, the substrate is a receptor, particularly a receptor with some portion exposed to the extracellular surface. Optionally, the substrate is a unique receptor subunit of a heteromeric receptor complex. In certain embodiments, the substrate is a biomolecules that is a component of a biomolecular accretion, such as an amyloid deposit or an atherosclerotic plaque. In certain embodiments, the substrate is an intracellular biomolecule, and in such instances, it may be desirable to use an adzyme that is able to enter the targeted cells, such as an adzyme that further comprises a transcytosis moiety that promotes transcytosis of the adzyme into the cell. In certain embodiments, the substrate is a biomolecule produced by a pathogen, such as a protozoan, a fungus, a bacterium or a virus. The substrate may be a prion protein. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin.

In a preferred embodiment, the substrate for an adzyme is TNFα. In the case of a direct adzyme, the targeting moiety binds to TNFα. Preferably, the catalytic domain comprises a protease that decreases TNFα activity. For example, the protease is may be selected from among: MT1-MMP; MMP12; tryptase; MT2-MMP; elastase; MMP7; chymotrypsin; and trypsin. The targeting moiety may be selected from among, a soluble portion of a TNFα receptor and a single chain antibody that binds to TNFα, although other targeting moieties are possible. A preferred targeting moiety is an sp55 portion of TNFα Receptor 1 (TNFR1).

In another preferred embodiment, the substrate for an adzyme is an interleukin-1, such as IL-1α or IL-1β. In the case of a direct adzyme, the targeting moiety binds to the interleukin-1 substrate. Preferably, the catalytic domain comprises a protease that decreases an IL-1 bioactivity.

In one aspect, the invention provides an adzyme for enzymatically altering a substrate, the adzyme comprising: a catalytic domain that catalyzes a chemical reaction converting said substrate to one or more products, and a targeting moiety that reversibly binds with an address site on said substrate or with an address site on a second molecule that occurs in functional proximity to the substrate, wherein said targeting moiety and said catalytic domain are heterologous with respect to each other, said targeting moiety, when provided separately, binds to the substrate, said catalytic domain, when provided separately, catalyzes the chemical reaction converting said substrate to one or more products, and said adzyme has one or more desirable properties, with respect to the reaction with said substrate. For example, in this aspect, the adzyme may have a potency at least 2 times greater than the catalytic domain or the targeting moiety alone, and preferably at least 3, 5, 10, 20 or more times greater than the potency of the catalytic domain or targeting moiety alone. The adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. The adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. The adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. The adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-3} sec^{-1}, k_{off} of 10^{-2} sec^{-1}, or greater. The adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. The adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the K_m of the catalytic domain alone. The adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, the adzyme will be designed so as to combine two or more of the above described properties.

In certain embodiments of an adzyme having one or more of such properties with respect to the reaction with the substrate molecule, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. The fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly or $SerGly_4$. In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said

substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

In certain embodiments of an adzyme having one or more of such properties, the adzyme is an immunoglobulin fusion, wherein the catalytic domain and the targeting moiety are joined, in a geometry consistent with effectiveness against substrate, to at least a portion of an immunoglobulin comprising a constant domain of an immunoglobulin. For example, the adzyme may comprise a first fusion protein and a second fusion protein, wherein the first fusion protein comprises a constant portion of an immunoglobulin heavy chain and a catalytic domain, and wherein the second fusion protein comprises a constant portion of an immunoglobulin heavy chain and a targeting domain that reversibly binds with an address site on or in functional proximity to the substrate. Preferably the immunoglobulin portions are Fc portions that dimerize by disulfide bonds.

In certain embodiments of an adzyme having one or more of such properties, a catalytic domain may include essentially any enzymatic domain that achieves the desired effect on a selected substrate. The catalytic domain may be selected so as to modify one or more pendant groups of said substrate. The substrate may include a chiral atom, and said catalytic domain may alter the ratio of stereoisomers. The catalytic domain may alter the level of post-translational modification of the polypeptide substrate, such as a glycosylation, phosphorylation, sulfation, fatty acid modification, alkylation, prenylation or acylation. Examples of enzymatic domains that may be selected include: a protease, an esterase, an amidase, a lactamase, a cellulase, an oxidase, an oxidoreductase, a reductase, a transferase, a hydrolase, an isomerase, a ligase, a lipase, a phospholipase, a phosphatase, a kinase, a sulfatase, a lysozyme, a glycosidase, a nuclease, an aldolase, a ketolase, a lyase, a cyclase, a reverse transcriptase, a hyaluronidase, an amylase, a cerebrosidase and a chitinase. Regardless of the type of catalytic domain, it may be desirable that the adzyme be resistant to autocatalysis (e.g., inter- or intra-molecular reactions), particularly at an adzyme concentration that is about equal to the concentration of adzyme in a solution to be administered to a subject. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate.

In certain preferred embodiments of an adzyme having one or more of the properties described above with respect to the reaction with the substrate, the adzyme includes a protease domain that, when active, cleaves at least one peptide bond of a polypeptide substrate. In general it will be desirable to design the adzyme such that it is resistant to cleavage by the protease catalytic domain. The protease domain may be generated as a zymogen (an inactive form) and then activated prior to use. The adzyme

may be purified from a cell culture in the presence of a reversible protease inhibitor, and such inhibitor may be included in any subsequent processing or storage activities.

In certain embodiments of an adzyme having one or more of the properties described above with respect to the reaction with the substrate, a targeting moiety may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting moiety will comprise a polypeptide or polypeptide complex, and particularly an antibody or polypeptide(s) including an antigen binding site of an antibody. For example, a targeting moiety may include a monoclonal antibody, an Fab and F(ab)2, an scFv, a heavy chain variable region and a light chain variable region. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In certain embodiments, the targeting moiety is a polyanionic or polycationic binding agent. Optionally, the targeting moiety is an oligonucleotide, a polysaccharide or a lectin. In certain embodiments, the substrate is a receptor, and the targeting moiety includes a ligand (or binding portion thereof) that binds to the receptor. In certain embodiments, the substrate is a ligand of a receptor, and the targeting moiety includes a ligand binding portion of the receptor, particularly a soluble ligand binding portion.

In certain embodiments of an adzyme having one or more of the properties described above with respect to the reaction with the substrate, the substrate is a biomolecule produced by a cell, such as a polypeptide, a polysaccharide, a nucleic acid, a lipid, or a small molecule. In certain embodiments, the substrate is a diffusible extracellular molecule, and preferably an extracellular signaling molecule that may act on an extracellular or intracellular receptor to triggers receptor-mediated cellular signaling. Optionally, the extracellular signaling molecule is an extracellular polypeptide signaling molecule, such as an inflammatory cytokine. In a preferred embodiment, the substrate is an interleukin-1 (e.g., IL-1α, IL-1β) or a TNF- α. In certain embodiments, the substrate is a polypeptide hormone, a growth factor and/or a cytokine, especially an inflammatory cytokine. Optionally, the adzyme acts to reduces a pro-inflammatory activity of a substrate. A substrate may be selected from is selected from the group consisting of four-helix bundle factors, EGF-like factors, insulin-like factors, β-trefoil factors and cysteine knot factors. In certain embodiments, the substrate is a receptor, particularly a receptor with some portion exposed to the extracellular surface. Optionally, the substrate is a unique receptor subunit of a heteromeric receptor complex. In certain embodiments, the biomolecule is a component of a biomolecular accretion, such as an amyloid deposit or an atherosclerotic plaque. In certain embodiments, the substrate is an intracellular biomolecule, and in such instances, it may be desirable to use an adzyme that is able to

enter the targeted cells, such as an adzyme that further comprises a transcytosis moiety that promotes transcytosis of the adzyme into the cell. In certain embodiments, the substrate is a biomolecule produced by a pathogen, such as a protozoan, a fungus, a bacterium or a virus. The substrate may be a prion protein. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin.

In a preferred embodiment of an adzyme having one or more of the properties described above with respect to the reaction with the substrate, the substrate for an adzyme is TNFα. In the case of a direct adzyme, the targeting moiety binds to TNFα. Preferably, the catalytic domain comprises a protease that decreases TNFα activity. For example, the protease is may be selected from among: MT1-MMP; MMP12; tryptase; MT2-MMP; elastase; MMP7; chymotrypsin; and trypsin. The targeting moiety may be selected from among, a soluble portion of a TNFα receptor and a single chain antibody that binds to TNFα, although other targeting moieties are possible. A preferred targeting moiety is an sp55 portion of TNFα Receptor 1 (TNFR1).

In another preferred embodiment of an adzyme having one or more of the properties described above with respect to the reaction with the substrate, the substrate for an adzyme is an interleukin-1, such as IL-1α or IL-1β. In the case of a direct adzyme, the targeting moiety binds to the interleukin-1 substrate. Preferably, the catalytic domain comprises a protease that decreases an IL-1 bioactivity.

In one aspect, the invention provides an adzyme for enzymatically altering a substrate, the adzyme comprising: a catalytic domain that catalyzes a chemical reaction converting said substrate to one or more products, and a targeting moiety that reversibly binds with an address site on said substrate or with an address site on a second molecule that occurs in functional proximity to the substrate, wherein the substrate is an extracellular signaling molecule, said targeting moiety and said catalytic domain are heterologous with respect to each other, said targeting moiety, when provided separately, binds to the substrate, said catalytic domain, when provided separately, catalyzes the chemical reaction converting said substrate to one or more products, and said chimeric protein construct is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate.

In certain embodiments of an adzyme that targets an extracellular signaling molecule, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a

recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly or $SerGly_4$. In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

In certain embodiments of an adzyme that targets an extracellular signaling molecule, the adzyme is an immunoglobulin fusion, wherein the catalytic domain and the targeting moiety are joined, in a geometry consistent with effectiveness against substrate, to at least a portion of an immunoglobulin comprising a constant domain of an immunoglobulin. For example, the adzyme may comprise a first fusion protein and a second fusion protein, wherein the first fusion protein comprises a constant portion of an immunoglobulin heavy chain and a catalytic domain, and wherein the second fusion protein comprises a constant portion of an immunoglobulin heavy chain and a targeting domain that reversibly binds with an address site on or in functional proximity to the substrate. Preferably the immunoglobulin portions are Fc portions that dimerize by disulfide bonds.

In certain embodiments of an adzyme that targets an extracellular signaling molecule, the adzyme is designed so as to have one or more desirable properties with respect to the reaction with said substrate. In many instances, such properties will be significant for achieving the desired effect of the adzyme on the substrate. For example, an adzyme may have a potency at least 2 times greater than the catalytic domain or the targeting moiety alone, and preferably at least 3, 5, 10, 20 or more times greater than the potency of the catalytic domain or targeting moiety alone. The adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. The adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. The adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. The adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-2} sec^{-1}, k_{off} of 10^{-2} sec^{-1}, or greater. The adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. The adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the

K_m of the catalytic domain alone. The adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, an adzyme will be designed so as to combine two or more of the above described properties.

In certain embodiments of an adzyme that targets an extracellular signaling molecule, a catalytic domain may include essentially any enzymatic domain that achieves the desired effect on a selected substrate. The catalytic domain may be selected so as to modify one or more pendant groups of said substrate. The substrate may include a chiral atom, and said catalytic domain may alter the ratio of stereoisomers. The catalytic domain may alter the level of post-translational modification of a polypeptide substrate, such as a glycosylation, phosphorylation, sulfation, fatty acid modification, alkylation, prenylation or acylation. Examples of enzymatic domains that may be selected include: a protease, an esterase, an amidase, a lactamase, a cellulase, an oxidase, an oxidoreductase, a reductase, a transferase, a hydrolase, an isomerase, a ligase, a lipase, a phospholipase, a phosphatase, a kinase, a sulfatase, a lysozyme, a glycosidase, a nuclease, an aldolase, a ketolase, a lyase, a cyclase, a reverse transcriptase, a hyaluronidase, an amylase, a cerebrosidase and a chitinase. Regardless of the type of catalytic domain, it may be desirable that the adzyme be resistant to autocatalysis (e.g., inter- or intra-molecular reactions), particularly at an adzyme concentration that is about equal to the concentration of adzyme in a solution to be administered to a subject. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate.

In certain preferred embodiments of an adzyme that targets an extracellular signaling molecule, the adzyme includes a protease domain that, when active, cleaves at least one peptide bond of a polypeptide substrate. In general it will be desirable to design the adzyme such that it is resistant to cleavage by the protease catalytic domain. The protease domain may be generated as a zymogen (an inactive form) and then activated prior to use. The adzyme may be purified from a cell culture in the presence of a reversible protease inhibitor, and such inhibitor may be included in any subsequent processing or storage activities.

In certain embodiments of an adzyme that targets an extracellular signaling molecule, a targeting moiety may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting moiety will comprise a polypeptide or polypeptide complex, and particularly an antibody or polypeptide(s)

including an antigen binding site of an antibody. For example, a targeting moiety may include a monoclonal antibody, an Fab and F(ab)2, an scFv, a heavy chain variable region and a light chain variable region. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In certain embodiments, the targeting moiety is a polyanionic or polycationic binding agent. Optionally, the targeting moiety is an oligonucleotide, a polysaccharide or a lectin. In certain embodiments, the substrate is a ligand of a receptor, and the targeting moiety includes a ligand binding portion of the receptor, particularly a soluble ligand binding portion.

In certain embodiments of an adzyme targeted to an extracellular signaling molecule, the substrate is preferably an extracellular signaling molecule that acts on an extracellular or intracellular receptor to triggers receptor-mediated cellular signaling. Optionally, the extracellular signaling molecule is an extracellular polypeptide signaling molecule, such as an inflammatory cytokine. In a preferred embodiment, the substrate is an interleukin-1 (e.g., IL-1α, IL-1β) or a TNF- α. In certain embodiments, the substrate is a polypeptide hormone, a growth factor and/or a cytokine, especially an inflammatory cytokine. Optionally, the adzyme acts to reduces a pro-inflammatory activity of a substrate. A substrate may be selected from is selected from the group consisting of four-helix bundle factors, EGF-like factors, insulin-like factors, β-trefoil factors and cysteine knot factors. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin.

In a preferred embodiment of an adzyme targeted to an extracellular signaling molecule, the substrate for the adzyme is TNFα. In the case of a direct adzyme, the targeting moiety binds to TNFα. Preferably, the catalytic domain comprises a protease that decreases TNFα activity. For example, the protease is may be selected from among: MT1-MMP; MMP12; tryptase; MT2-MMP; elastase; MMP7; chymotrypsin; and trypsin. The targeting moiety may be selected from among, a soluble portion of a TNFα receptor and a single chain antibody that binds to TNFα, although other targeting moieties are possible. A preferred targeting moiety is an sp55 portion of TNFα Receptor 1 (TNFR1).

In another preferred embodiment of an adzyme targeted to an extracellular signaling molecule, the substrate for an adzyme is an interleukin-1, such as IL-1α or IL-1β. In the case of a direct adzyme, the targeting moiety binds to the interleukin-1 substrate. Preferably, the catalytic domain comprises a protease that decreases an IL-1 bioactivity.

In one aspect, the invention provides adzymes for enzymatically altering a substrate, the adzyme comprising a polypeptide comprising: a catalytic domain that

catalyzes a chemical reaction converting said substrate to one or more products, a targeting domain that reversibly binds with an address site on said substrate or with an address site on a second molecule that occurs in functional proximity to the substrate, and a linker joining said catalytic domain and said targeting domain, wherein said substrate is a receptor, said targeting moiety and said catalytic domain are heterologous with respect to each other, said targeting domain, when provided separately, binds to the substrate, said catalytic domain, when provided separately, catalyzes the chemical reaction converting said substrate to one or more products, and said chimeric protein construct is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate.

In certain embodiments of an adzyme that targets a receptor, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly or $SerGly_4$. In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

In certain embodiments of an adzyme that targets a receptor, the adzyme is an immunoglobulin fusion, wherein the catalytic domain and the targeting moiety are joined, in a geometry consistent with effectiveness against substrate, to at least a portion of an immunoglobulin comprising a constant domain of an immunoglobulin. For example, the adzyme may comprise a first fusion protein and a second fusion protein, wherein the first fusion protein comprises a constant portion of an immunoglobulin heavy chain and a catalytic domain, and wherein the second fusion protein comprises a constant portion of an immunoglobulin heavy chain and a targeting domain that reversibly binds with an address site on or in functional proximity to the substrate. Preferably the immunoglobulin portions are Fc portions that dimerize by disulfide bonds.

In certain embodiments of an adzyme that targets a receptor, the adzyme is designed so as to have one or more desirable properties adzyme has one or more desirable

properties, with respect to the reaction with said substrate. In many instances, such properties will be significant for achieving the desired effect of the adzyme on the substrate. For example, an adzyme may have a potency at least 2 times greater than the catalytic domain or the targeting moiety alone, and preferably at least 3, 5, 10, 20 or more times greater than the potency of the catalytic domain or targeting moiety alone. The adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. The adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. The adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. The adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-2} sec^{-1}, k_{off} of 10^{-2} sec^{-1}, or greater. The adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. The adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the K_m of the catalytic domain alone. The adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, an adzyme will be designed so as to combine two or more of the above described properties.

In certain embodiments of an adzyme that targets a receptor, a catalytic domain may include essentially any enzymatic domain that achieves the desired effect on a selected substrate. The catalytic domain may be selected so as to modify one or more pendant groups of said substrate. The substrate may include a chiral atom, and said catalytic domain may alter the ratio of stereoisomers. The catalytic domain may alter the level of post-translational modification of a polypeptide substrate, such as a glycosylation, phosphorylation, sulfation, fatty acid modification, alkylation, prenylation or acylation. Examples of enzymatic domains that may be selected include: a protease, an esterase, an amidase, a lactamase, a cellulase, an oxidase, an oxidoreductase, a reductase, a transferase, a hydrolase, an isomerase, a ligase, a lipase, a phospholipase, a phosphatase, a kinase, a sulfatase, a lysozyme, a glycosidase, a nuclease, an aldolase, a ketolase, a lyase, a cyclase, a reverse transcriptase, a hyaluronidase, an amylase, a cerebrosidase and a chitinase. Regardless of the type of catalytic domain, it may be desirable that the adzyme be resistant to autocatalysis (e.g., inter- or intra-molecular reactions), particularly at an adzyme concentration that is about equal to the concentration of adzyme in a solution to be administered to a subject. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate.

In certain preferred embodiments of an adzyme that targets a receptor, the adzyme includes a protease domain that, when active, cleaves at least one peptide bond of a polypeptide substrate. In general it will be desirable to design the adzyme such that it is resistant to cleavage by the protease catalytic domain. The protease domain may be generated as a zymogen (an inactive form) and then activated prior to use. The adzyme may be purified from a cell culture in the presence of a reversible protease inhibitor, and such inhibitor may be included in any subsequent processing or storage activities.

In certain embodiments of an adzyme that targets a receptor, a targeting moiety may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting moiety will comprise a polypeptide or polypeptide complex, and particularly an antibody or polypeptide(s) including an antigen binding site of an antibody. For example, a targeting moiety may include a monoclonal antibody, an Fab and F(ab)2, an scFv, a heavy chain variable region and a light chain variable region. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In certain embodiments, the targeting moiety is a polyanionic or polycationic binding agent. Optionally, the targeting moiety is an oligonucleotide, a polysaccharide or a lectin. The targeting moiety may include a ligand (or binding portion thereof) that binds to the receptor.

In certain embodiments of an adzyme that targets a receptor, the substrate is a receptor with some portion exposed to the extracellular surface. Optionally, the substrate is a unique receptor subunit of a heteromeric receptor complex. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin.

In a preferred embodiment of an adzyme targeted to a receptor, the substrate for the adzyme is a TNFα receptor, such as TNFR1 or TNFR2. In the case of a direct adzyme, the targeting moiety binds to the receptor. Preferably, the catalytic domain comprises a protease that decreases the TNFα stimulated activity of the receptor. The targeting moiety may be selected from among, a receptor binding portion of TNFα and a single chain antibody that binds to the receptor, although other targeting moieties are possible.

In another preferred embodiment of an adzyme targeted to a receptor, the substrate for an adzyme is an interleukin-1 receptor (IL-1R). In the case of a direct adzyme, the targeting moiety binds to the IL-1R. Preferably, the catalytic domain comprises a protease that decreases an IL-1R bioactivity.

In a further aspect, the invention provides an adzyme for enzymatically altering a substrate, the adzyme comprising: a catalytic domain that catalyzes a chemical reaction converting said substrate to one or more products, and a targeting moiety that reversibly binds with an address site on said substrate or with an address site on a second molecule that occurs in functional proximity to the substrate, wherein one or more of said products is an antagonist of an activity of said substrate.

In certain embodiments of an adzyme that generates an antagonist of the substrate, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly or $SerGly_4$. In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

In certain embodiments of an adzyme that generates an antagonist of the substrate, the adzyme is an immunoglobulin fusion, wherein the catalytic domain and the targeting moiety are joined, in a geometry consistent with effectiveness against substrate, to at least a portion of an immunoglobulin comprising a constant domain of an immunoglobulin. For example, the adzyme may comprise a first fusion protein and a second fusion protein, wherein the first fusion protein comprises a constant portion of an immunoglobulin heavy chain and a catalytic domain, and wherein the second fusion protein comprises a constant portion of an immunoglobulin heavy chain and a targeting domain that reversibly binds with an address site on or in functional proximity to the substrate. Preferably the immunoglobulin portions are Fc portions that dimerize by disulfide bonds.

In certain embodiments of an adzyme that generates an antagonist of the substrate, the adzyme is designed so as to have one or more desirable properties adzyme has one or more desirable properties, with respect to the reaction with said substrate. In many instances, such properties will be significant for achieving the desired effect of the adzyme on the substrate. For example, an adzyme may have a potency at least 2 times greater than the catalytic domain or the targeting moiety alone, and preferably at least 3, 5, 10, 20 or

more times greater than the potency of the catalytic domain or targeting moiety alone. The adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. The adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. The adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. The adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-2} sec^{-1}, k_{off} of 10^{-2} sec^{-1}, or greater. The adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. The adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the K_m of the catalytic domain alone. The adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, an adzyme will be designed so as to combine two or more of the above described properties.

In certain embodiments of an adzyme that generates an antagonist of the substrate, a catalytic domain may include essentially any enzymatic domain that achieves the desired effect on a selected substrate. The catalytic domain may be selected so as to modify one or more pendant groups of said substrate. The substrate may include a chiral atom, and said catalytic domain may alter the ratio of stereoisomers. The catalytic domain may alter the level of post-translational modification of a polypeptide substrate, such as a glycosylation, phosphorylation, sulfation, fatty acid modification, alkylation, prenylation or acylation. Examples of enzymatic domains that may be selected include: a protease, an esterase, an amidase, a lactamase, a cellulase, an oxidase, an oxidoreductase, a reductase, a transferase, a hydrolase, an isomerase, a ligase, a lipase, a phospholipase, a phosphatase, a kinase, a sulfatase, a lysozyme, a glycosidase, a nuclease, an aldolase, a ketolase, a lyase, a cyclase, a reverse transcriptase, a hyaluronidase, an amylase, a cerebrosidase and a chitinase. Regardless of the type of catalytic domain, it may be desirable that the adzyme be resistant to autocatalysis (e.g., inter- or intra-molecular reactions), particularly at an adzyme concentration that is about equal to the concentration of adzyme in a solution to be administered to a subject. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate. In certain embodiment, the adzyme acts on the substrate such that a product of the chemical reaction is an antagonist of the substrate.

In certain preferred embodiments of an adzyme that generates an antagonist of the substrate, the adzyme includes a protease domain that, when active, cleaves at least one peptide bond of a polypeptide substrate. In general it will be desirable to design the

adzyme such that it is resistant to cleavage by the protease catalytic domain. The protease domain may be generated as a zymogen (an inactive form) and then activated prior to use. The adzyme may be purified from a cell culture in the presence of a reversible protease inhibitor, and such inhibitor may be included in any subsequent processing or storage activities.

In certain embodiments of an adzyme that generates an antagonist of the substrate, a targeting moiety may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting moiety will comprise a polypeptide or polypeptide complex, and particularly an antibody or polypeptide(s) including an antigen binding site of an antibody. For example, a targeting moiety may include a monoclonal antibody, an Fab and F(ab)2, an scFv, a heavy chain variable region and a light chain variable region. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In certain embodiments, the targeting moiety is a polyanionic or polycationic binding agent. Optionally, the targeting moiety is an oligonucleotide, a polysaccharide or a lectin. The targeting moiety may include a ligand (or binding portion thereof) that binds to the receptor.

In certain embodiments of an adzyme that generates an antagonist of the substrate, the substrate is a biomolecule produced by a cell, such as a polypeptide, a polysaccharide, a nucleic acid, a lipid, or a small molecule. In certain embodiments, the substrate is a diffusible extracellular molecule, and preferably an extracellular signaling molecule that may act on an extracellular or intracellular receptor to triggers receptor-mediated cellular signaling. Optionally, the extracellular signaling molecule is an extracellular polypeptide signaling molecule, such as an inflammatory cytokine. In a preferred embodiment, the substrate is an interleukin-1 (e.g., IL-1α, IL-1β) or a TNF- α. In certain embodiments, the substrate is a polypeptide hormone, a growth factor and/or a cytokine, especially an inflammatory cytokine. Optionally, the adzyme acts to reduces a pro-inflammatory activity of a substrate. A substrate may be selected from is selected from the group consisting of four-helix bundle factors, EGF-like factors, insulin-like factors, β-trefoil factors and cysteine knot factors. In certain embodiments, the substrate is a receptor, particularly a receptor with some portion exposed to the extracellular surface. Optionally, the substrate is a unique receptor subunit of a heteromeric receptor complex. In certain embodiments, the biomolecule is a component of a biomolecular accretion, such as an amyloid deposit or an atherosclerotic plaque. In certain embodiments, the substrate is an intracellular biomolecule, and in such instances, it may be desirable to use an adzyme that is able to enter the targeted cells, such as an adzyme that further comprises a transcytosis

moiety that promotes transcytosis of the adzyme into the cell. In certain embodiments, the substrate is a biomolecule produced by a pathogen, such as a protozoan, a fungus, a bacterium or a virus. The substrate may be a prion protein. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin.

In a preferred embodiment, the substrate for an adzyme is TNFα. In the case of a direct adzyme, the targeting moiety binds to TNFα. Preferably, the catalytic domain comprises a protease that decreases TNFα activity. For example, the protease is may be selected from among: MT1-MMP; MMP12; tryptase; MT2-MMP; elastase; MMP7; chymotrypsin; and trypsin. The targeting moiety may be selected from among, a soluble portion of a TNFα receptor and a single chain antibody that binds to TNFα, although other targeting moieties are possible. A preferred targeting moiety is an sp55 portion of TNFα Receptor 1 (TNFR1).

In another preferred embodiment, the substrate for an adzyme is an interleukin-1, such as IL-1α or IL-1β. In the case of a direct adzyme, the targeting moiety binds to the interleukin-1 substrate. Preferably, the catalytic domain comprises a protease that decreases an IL-1 bioactivity.

In one aspect, the invention provides adzymes for enzymatically altering a substrate, the adzyme comprising: a catalytic domain that cleaves at least one peptide bond of said substrate to produce one or more products, and a polypeptide targeting domain that reversibly binds with an address site on said substrate or with an address site on a second molecule that occurs in functional proximity to the substrate, wherein said adzyme is resistant to cleavage by the catalytic domain, said targeting moiety, when provided separately, binds to the substrate, said catalytic domain, when provided separately, cleaves at least one peptide bond of said substrate to produce one or more products, and said chimeric protein construct is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate.

In certain embodiments of a proteolytic adzyme, a catalytic domain and a targeting domain of the adzyme are joined by a polypeptide and linker to form a fusion protein. A fusion protein may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly or $SerGly_4$. In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting

moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety.

In certain embodiments of a proteolytic adzyme, the adzyme is an immunoglobulin fusion, wherein the catalytic domain and the targeting moiety are joined, in a geometry consistent with effectiveness against substrate, to at least a portion of an immunoglobulin comprising a constant domain of an immunoglobulin. For example, the adzyme may comprise a first fusion protein and a second fusion protein, wherein the first fusion protein comprises a constant portion of an immunoglobulin heavy chain and a catalytic domain, and wherein the second fusion protein comprises a constant portion of an immunoglobulin heavy chain and a targeting domain that reversibly binds with an address site on or in functional prosimity to the substrate. Preferably the immunoglobulin portions are Fc portions that dimerize by disulfide bonds.

In certain embodiments of a proteolytic adzyme, the adzyme is designed so as to have one or more desirable properties adzyme has one or more desirable properties, with respect to the reaction with said substrate. In many instances, such properties will be significant for achieving the desired effect of the adzyme on the substrate. For example, an adzyme may have a potency at least 2 times greater than the catalytic domain or the targeting moiety alone, and preferably at least 3, 5, 10, 20 or more times greater than the potency of the catalytic domain or targeting moiety alone. The adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. The adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. The adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. The adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-2} sec^{-1}, k_{off} of 10^{-2} sec^{-1}, or greater. The adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. The adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the K_m of the catalytic domain alone. The adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, an adzyme will be designed so as to combine two or more of the above described properties.

In certain embodiments of the proteolytic adzyme, the substrate is a polypeptide produced by a cell. In certain embodiments, the substrate is a diffusible extracellular

polypeptide, and preferably an extracellular polypeptide signaling molecule that may act on an extracellular or intracellular receptor to triggers receptor-mediated cellular signaling. Optionally, the substrate is an inflammatory cytokine. In a preferred embodiment, the substrate is an interleukin-1 (e.g., IL-1α, IL-1β) or a TNF- α. In certain embodiments, the substrate is a polypeptide hormone, a growth factor and/or a cytokine, especially an inflammatory cytokine. Optionally, the adzyme acts to reduces a pro-inflammatory activity of a substrate. A substrate may be selected from is selected from the group consisting of four-helix bundle factors, EGF-like factors, insulin-like factors, β-trefoil factors and cysteine knot factors. In certain embodiments, the substrate is a receptor, particularly a receptor with some portion exposed to the extracellular surface. Optionally, the substrate is a unique receptor subunit of a heteromeric receptor complex. In certain embodiments, the biomolecule is a component of a biomolecular accretion, such as an amyloid deposit or an atherosclerotic plaque. In certain embodiments, the substrate is an intracellular biomolecule, and in such instances, it may be desirable to use an adzyme that is able to enter the targeted cells, such as an adzyme that further comprises a transcytosis moiety that promotes transcytosis of the adzyme into the cell. In certain embodiments, the substrate is a biomolecule produced by a pathogen, such as a protozoan, a fungus, a bacterium or a virus. The substrate may be a prion protein. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin.

In general, with a proteolytic adzyme, it will be desirable to design the adzyme such that it is resistant to cleavage by the protease catalytic domain. The protease domain may be generated as a zymogen (an inactive form) and then activated prior to use. The adzyme may be purified from a cell culture in the presence of a reversible protease inhibitor, and such inhibitor may be included in any subsequent processing or storage activities.

In certain embodiments of a proteolytic adzyme, a targeting moiety may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting moiety will comprise a polypeptide or polypeptide complex, and particularly an antibody or polypeptide(s) including an antigen binding site of an antibody. For example, a targeting moiety may include a monoclonal antibody, an Fab and F(ab)2, an scFv, a heavy chain variable region and a light chain variable region. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In certain embodiments, the targeting moiety is a polyanionic or polycationic binding agent.

Optionally, the targeting moiety is an oligonucleotide, a polysaccharide or a lectin. In certain embodiments, the substrate is a receptor, and the targeting moiety includes a ligand (or binding portion thereof) that binds to the receptor. In certain embodiments, the substrate is a ligand of a receptor, and the targeting moiety includes a ligand binding portion of the receptor, particularly a soluble ligand binding portion.

In a preferred embodiment, the substrate for an adzyme is TNFα. In the case of a direct adzyme, the targeting moiety binds to TNFα. Preferably, the catalytic domain comprises a protease that decreases TNFα activity. For example, the protease is may be selected from among: MT1-MMP; MMP12; tryptase; MT2-MMP; elastase; MMP7; chymotrypsin; and trypsin. The targeting moiety may be selected from among, a soluble portion of a TNFα receptor and a single chain antibody that binds to TNFα, although other targeting moieties are possible. A preferred targeting moiety is an sp55 portion of TNFα Receptor 1 (TNFR1).

In another preferred embodiment, the substrate for an adzyme is an interleukin-1, such as IL-1α or IL-1β. In the case of a direct adzyme, the targeting moiety binds to the interleukin-1 substrate. Preferably, the catalytic domain comprises a protease that decreases an IL-1 bioactivity.

In one aspect, the invention provides adzymes for enzymatically altering a substrate, the adzyme comprising a polypeptide comprising: a catalytic domain that catalyzes a chemical reaction converting said substrate to one or more products, a targeting domain that reversibly binds with an address site on said substrate or with an address site on a second molecule that occurs in functional proximity to the substrate, and a linker joining said catalytic domain and said targeting domain, wherein said substrate is an extracellular polypeptide signaling molecule, said targeting moiety and said catalytic domain are heterologous with respect to each other, said targeting domain, when provided separately, binds to said substrate, said catalytic domain, when provided separately, catalyzes the chemical reaction converting said substrate to one or more products, and said adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate.

In certain aspects, the invention provides an adzyme for inhibiting receptor-mediated signaling activity of an extracellular substrate polypeptide, the adzyme being a fusion protein comprising a protease domain that catalyzes the proteolytic cleavage of at least one peptide bond of the substrate polypeptide so as to inhibit the receptor-mediated signaling activity of the polypeptide, and a targeting domain that reversibly binds with an address site on said substrate polypeptide, wherein said targeting domain and said protease domain are discrete and heterologous with respect to each other. Optionally, the adzyme is resistant to cleavage by said protease domain. Optionally, the protease domain is a

zymogen. Optionally, the protease domain is selected from among: a serine proteinase, a cysteine protease, a threonine protease, an aspartate protease and a metalloproteinase. Optionally, the adzyme is purified from a cell culture in the presence of a reversible protease inhibitor that inhibits the protease activity of the protease domain. In certain embodiments, the adzyme has one or more properties, with respect to the reaction with said substrate In many instances, such properties will be significant for achieving the desired effect of the adzyme on the substrate. For example, an adzyme may have a potency at least 2 times greater than the catalytic domain or the targeting moiety alone, and preferably at least 3, 5, 10, 20 or more times greater than the potency of the catalytic domain or targeting moiety alone. The adzyme may have a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, and optionally a k_{on} of 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$, 10^6 $M^{-1}s^{-1}$, 10^7 $M^{-1}s^{-1}$ or greater. The adzyme may have a k_{cat} of 0.1 sec^{-1} or greater, and optionally a k_{cat} of 1 sec^{-1}, 10 sec^{-1}, 50 sec^{-1} or greater. The adzyme may have a K_D that is at least 5, 10, 25, 50 or 100 or more fold less than the K_m of the catalytic domain. The adzyme may have a k_{off} of 10^{-4} sec^{-1} or greater, and optionally a k_{off} of 10^{-2} sec^{-1}, k_{off} of 10^{-2} sec^{-1}, or greater. The adzyme may have a catalytic efficiency that is at least 5 fold greater than the catalytic efficiency of the catalytic domain alone, and optionally a catalytic efficiency that is at least 10 fold, 20 fold, 50 fold or 100 fold greater than that of the catalytic domain. The adzyme may have a K_m at least 5 fold, 10 fold, 20 fold, 50 fold, or 100 fold less than the K_m of the catalytic domain alone. The adzyme may have an effective substrate concentration that is at least 5 fold, 10 fold, 20 fold, 50 fold or 100 fold greater than the actual substrate concentration. In certain preferred embodiments, an adzyme will be designed so as to combine two or more of the above described properties. Optionally, the substrate is an inflammatory cytokine. In a preferred embodiment, the substrate is an interleukin-1 (e.g., IL-1α, IL-1β) or a TNF- α. In certain embodiments, the substrate is a polypeptide hormone, a growth factor and/or a cytokine, especially an inflammatory cytokine. Optionally, the adzyme acts to reduces a pro-inflammatory activity of a substrate. A substrate may be selected from is selected from the group consisting of four-helix bundle factors, EGF-like factors, insulin-like factors, β-trefoil factors and cysteine knot factors. In a preferred embodiment, the substrate is endogenous to a human patient. In such an embodiment, the adzyme is preferably effective against the substrate in the presence of physiological levels of an abundant human serum protein, such as, serum albumins or an abundant globin. The fusion protein adzymes may be generated in a variety of ways, including chemical coupling and cotranslation. In a preferred embodiment, the fusion protein is a cotranslational fusion protein encoded by a recombinant nucleic acid. In certain embodiments the linker for the fusion protein is an unstructured peptide. Optionally, the linker includes one or more repeats of Ser_4Gly or $SerGly_4$. In preferred embodiments, the linker is selected to provide steric geometry between said catalytic domain and said

targeting moiety such that said adzyme is more effective against the substrate than either the catalytic domain or targeting moiety alone. For example, the linker may be selected such that the adzyme is more potent than said catalytic domain or targeting moiety with respect to the reaction with said substrate. The linker may be selected such that the targeting moiety presents the substrate to the enzymatic domain at an effective concentration at least 5 fold greater than would be present in the absence of the targeting moiety. A targeting domain may include essentially any molecule or assembly of molecules that binds to the address site (e.g., on the substrate in the case of direct adzymes or on a molecule that occurs in functional proximity to the substrate, in the case of proximity adzymes). In many embodiments, a targeting domain will comprise an antigen binding site of an antibody, such as a single chain antibody. Optionally, the targeting moiety is an artificial protein or peptide sequence engineered to bind to the substrate. In a preferred embodiment, the substrate for the adzyme is TNFα. In the case of a direct adzyme, the targeting moiety binds to TNFα. Preferably, the catalytic domain comprises a protease that decreases TNFα activity. For example, the protease is may be selected from among: MT1-MMP; MMP12; tryptase; MT2-MMP; elastase; MMP7; chymotrypsin; and trypsin. The targeting moiety may be selected from among, a soluble portion of a TNFα receptor and a single chain antibody that binds to TNFα, although other targeting moieties are possible. A preferred targeting moiety is an sp55 portion of TNFα Receptor 1 (TNFR1). In another preferred embodiment, the substrate for the adzyme is an interleukin-1, such as IL-1α or IL-1β. In the case of a direct adzyme, the targeting moiety binds to the interleukin-1 substrate. Preferably, the catalytic domain comprises a protease that decreases an IL-1 bioactivity.

In one aspect, the invention provides an adzyme for inhibiting receptor-mediated signaling activity of an extracellular substrate polypeptide, the adzyme being an immunoglobulin fusion complex. For example, such an adzyme may comprise: a first fusion protein bound to a second fusion protein, wherein the first fusion protein comprises a constant portion of an immunoglobulin heavy chain and a protease domain that catalyzes the proteolytic cleavage of at least one peptide bond of the substrate polypeptide so as to inhibit the receptor-mediated signaling activity of the polypeptide, and wherein the second fusion protein comprises a constant portion of an immunoglobulin heavy chain and a targeting domain that reversibly binds with an address site on said substrate polypeptide, wherein said targeting domain and said protease domain are discrete and heterologous with respect to each other.

In certain aspects, the invention provides adzyme preparations for use in a desired application, such as a therapeutic use, an industrial use, an environmental use or in a microfabrication. Such preparations may be termed adzyme preparations. In certain embodiments, the invention provides an adzyme preparation for therapeutic use in a

human patient, the preparation comprising any adzyme disclosed herein. Optionally, the preparation further comprising a pharmaceutically effective carrier. Optionally, the adzyme preparation is formulated such that autocatalytic modification of the adzyme is inhibited. Optionally, the adzyme comprises a catalytic domain that is a protease, and in certain embodiments, the preparation comprises a reversible inhibitor of said protease, preferably a reversible inhibitor that is safe for administration to a human patient. Optionally, an adzyme preparation for therapeutic use is substantially pyrogen free. An adzyme preparation may be packaged along with instructions for use. For example, an adzyme preparation for therapeutic use may be packaged with instructions for administration to a patient.

In certain aspects, the invention provides methods for making a medicament for use in treating a disorder that is associated with an activity of the substrate of any adzyme disclosed herein, the method comprising formulating the adzyme for administration to a patient, preferably a human patient. In certain embodiments, the invention provides a method of making a medicament for use in treating an inflammatory or allergic disorder, the method comprising formulating an adzyme for administration to a human patient in need thereof, wherein the substrate of the adzyme is an inflammatory cytokine.

In certain aspects, the invention provides methods of treating a disorder that is associated with an activity of the substrate of an adzyme, the method comprising administering a therapeutically effective dose of an adzyme to a human patient in need thereof. In certain embodiments, an adzyme may be used in a method of treating an inflammatory of allergic disorder, the method comprising administering a therapeutically effective dose of an adzyme to a human patient in need thereof, wherein the substrate of the adzyme is an inflammatory cytokine.

In certain aspects, the invention provides nucleic acids encoding any of the various polypeptide portions of an adzyme, and particularly recombinant nucleic acids encoding a fusion protein adzyme. Such nucleic acids may be incorporated into an expression vector wherein the expression vector directs expression of the adzyme in a suitable host cell. The invention further provides cells comprising such nucleic acids and vectors. In certain embodiments, the invention provides cells comprising a first nucleic acid comprising a first coding sequence and a second nucleic acid comprising a second coding sequence, wherein the first coding sequence encodes a first fusion protein comprising an immunoglobulin heavy chain and a catalytic domain, and wherein the second coding sequence encodes a second fusion protein comprising an immunoglobulin heavy chain and a targeting domain. Preferably, such as cell, in appropriate culture conditions, secretes an adzyme comprising an Fc fusion protein construct that is a dimer of the first fusion protein and the second fusion protein.

In certain aspects, the invention provides methods for manufacturing an adzyme. Such methods may include expression of polypeptide components in cells. Such methods may include chemical joining of various adzyme components. In one embodiment, a method comprises culturing a cell having an expression vector for producing a fusion protein adzyme in conditions that cause the cell to produce the adzyme encoded by the expression vector; and purifying the adzyme to substantial purity. In one embodiments, a method comprises culturing a cell designed to produce an immunoglobulin fusion in conditions that cause the cell to produce the adzyme encoded by the expression vector; and purifying the adzyme to substantial purity. In certain embodiments, purifying an adzyme to substantial purity includes the use of a reversible inhibitor that inhibits autocatalytic activity of the catalytic domain, and particularly, wherein the catalytic domain of the adzyme is a protease domain, and wherein purifying the adzyme to substantial purity includes the use of a reversible protease inhibitor that inhibits the protease activity of the catalytic domain.

In further aspects, the invention provides methods for designing and producing adzymes with desirable properties, and methods for operating a business that involves designing and selling adzymes with desirable properties, such as therapeutically effective adzymes.

The embodiments and practices of the present invention, other embodiments, and their features and characteristics, will be apparent from the description, figures and claims that follow.

Brief Description of the Drawings

Figures 1A-1J are schematic representations of the structure of a series of different exemplary constructs embodying the invention. The boxes represent moieties having binding or catalytic properties, and can be embodied as true protein domains, *i.e.*, bonded sequences of amino acids forming structures characterized by folding of the peptide chain into alpha helices, beta pleated sheets, random coils, etc., to form separate binding surfaces or enzymatically active sites, and including catalytic moieties (CAT), address moieties (ADD), and protein domains serving to associate these parts together in various operative configurations. Lines connecting boxes represent a covalent bond linking together amino acid sequence defining the respective functional regions, or linkers comprising, for example, a flexible linear linker such as a string of peptide bonded amino acids or a poly(ethylene glycol) chain. Lines between boxes represent non covalent, reversible attachments wherein the parts are held together by a combination of forces such

as hydrogen bonding, hydrophobic-hydrophobic interaction, opposite charge matching, etc., for example, ligand-receptor interactions.

Figure 1K is a schematic diagram illustrating the basic concept of a contingent adzyme.

Figures 2A-2J are cartoons illustrating various exemplary embodiments of adzyme constructs at various types of targeted biomolecules in position to initiate an enzymatic reaction on the substrate site of the target. The address is designated as AD, the catalytic domain as CD.

Figures 3A-3G are cartoons illustrating various exemplary embodiments of contingent adzyme constructs in the absence of and in the vicinity of their respective intended targeted biomolecules.

Figure 4 is a cartoon illustrating components of a pre thrombin scFvαHA adzyme.

Figure 5 is electrophoretic analysis of purified model adzyme.

Figure 6 is Western blot analysis of model adzyme activated using Factor Xa.

Figure 7 shows proteolytic activity of thrombin and model adzyme before and after activation on standard thrombin tripeptide substrate.

Figure 8 shows that enhanced adzyme activity is driven by the presence of an address domain.

Figure 9 shows that enhanced adzyme activity requires cotranslational linkage of the domains.

Figure 10 shows proteolytic inactivation of TNFα cytotoxicity.

Figure 11 shows that soluble TNFα receptor p55 address domain binds TNFα.

Figure 12 is a representative expression of several adzyme constrcuts as analyzed by Western blotting with anti-myc antibody. Lane 1: trypsinogen expressed in the absence of stabilizing benzamidine, Lane 2: trypsinogen, Lane 3: trypsinogen-0aa-sp55, Lane 4: trypsinogen-20aa-sp55; Lane 5: trypsinogen-3aa-sp55, Lane 6: sp55. Material in lanes 2 through 6 was expressed in the presence of 1 mM benzamidine.

Figure 13 shows a snapshot of representative experiments where the fluorescence detected at the end of 2 hours of incubation is compared for the different recombinant adzymes and other control proteins.

Figure 14 shows normalization of trypsin activities.

Figure 15 shows detection of TNFα binding of adzymes by ELISA.

Figure 16 shows kinetic model results comparing the performance of an adzyme, an address, and an enzyme.

Detailed Description of the Invention

I. Overview

The invention provides a new class of engineered protein constructs, referred to herein as "adzymes", as well as methods and compositions related to the use and production of adzymes. Adzymes are chimeric protein constructs that join one or more catalytic domains with one or more targeting moieties (or "addresses"). A catalytic domain of an adzyme has an enzymatically active site that catalyzes a reaction converting a pre-selected substrate (the "target" or "targeted substrate") into one or more products, such as by cleavage, chemical modifications (transformations) or isomerization. Generally, the catalytic domain is selected such that one or more of the product(s) of the adzyme-mediated reaction have a qualitatively or quantitatively different activity relative to the selected substrate. Merely to illustrate, the adzyme may alter such functional characteristics of a selected substrate as affinity, potency, selectivity, solubility, immunogenicity, half-life, clearance (such as by renal or hepatic function), biodistribution or other pharmacokinetic properties. In certain instances, the product of an adzyme-mediated reaction is itself an antagonist of an activity of the selected substrate.

The targeting moiety (or "address") is a moiety capable of recognizing and reversibly binding to a pre-determined "address binding site" (also herein "address site"), such as, for example, a soluble or membrane-bound biomolecules, or a component of a biomolecular accretion (*e.g.*, a plaque or other insoluble protein-containing aggregate). In certain types of adzymes (termed "direct adzymes"), the targeting moiety binds to the target molecule. In certain types of adzymes (termed "proximity adzymes") the targeting moiety binds to a molecule that tends to occur in functional proximity to the target. The term "moiety" should be understood as including single molecules or portions thereof (*e.g.*, a polypeptide or sugar that binds to the address binding site), as well as combinations of molecules (*e.g.*, an antibody that binds to an address binding site).

In an adzyme, at least one targeting moiety is operatively associated with at least one catalytic domain. An adzyme may be a single polypeptide chain (e.g., a fusion protein) or an assembly of polypeptide chains and/or other molecules that are joined through covalent or non-covalent bonds. Regardless of how the constituent portions of an adzyme are associated, at least one targeting moiety and one catalytic domain should be operatively associated. The term "operatively associated", as used herein to describe the relationship between a catalytic domain and a targeting moiety, means that the

effectiveness of the associated catalytic domain and targeting moiety in chemically altering or otherwise affecting the activity of the pre-selected substrate is greater than the effectiveness of either the targeting moiety or the catalytic domain alone, and also greater than the effectiveness of both the targeting moiety and the catalytic domain when provided in combination but not in association with each other (*e.g.*, where the target is simultaneously contacted with both a discrete catalytic domain and a discrete targeting moiety). As described below, the adzyme may include other components as well, such as linkers, moieties that influence stability or biodistribution, and the like.

The effectiveness of an adzyme relative to its constituent parts may be assessed in a variety of ways. For example, effectiveness may be assessed in terms of potency of the adzyme, as compared to its component parts, to affect a biological activity of the pre-selected substrate. As another example, effectiveness may be assessed in terms of a comparison of kinetic or equilibrium constants that describe the reaction between the adzyme and the pre-selected substrate to those that apply to the reaction between the component parts and the targeted substrate. In embodiments where an adzyme is intended for use in a mammal, at least one catalytic domain and at least one targeting moiety of an adzyme will be associated such that these portions are operatively associated under physiological conditions (e.g., in whole blood, serum, cell culture conditions, or phosphate buffered saline solution, pH 7). Where the adzyme is intended for other purposes (e.g., the modification of an environmental pollutant or the modification of a component of a molecular reaction), at least one catalytic domain and at least one targeting moiety of an adzyme will be associated such that these portions are operatively associated under the expected or desired reaction conditions.

Merely to illustrate, an adzyme may comprise a catalytic domain that cleaves or otherwise modifies TNF-α, converting it into one or more products having reduced activity, no activity or antagonist activity, thereby ameliorating a disease state associated with TNF-α, such as rheumatoid arthritis or other conditions associated with TNF-α activity.

While not wishing to be bound to any particular mechanism of action, it is expected that a targeting moiety will bind to the pre-selected targeted substrate (direct adzyme) or to another molecule that occurs in the same vicinity as the pre-selected targeted substrate (proximity adzyme), and thereby functions to increase the concentration of the catalytic domain at or near the targeted substrate. In this way, the adzyme is self-concentrating at or in the vicinity of a targeted substrate and has an enhanced effectiveness for reacting with and altering the activity of the targeted substrate, relative to the catalytic or binding domains alone. As a consequence to the improved effectiveness of the targeted

reaction, the adzyme has a greater selectivity and/or catalytic efficiency for the targeted substrate as compared to other non-targeted (potential) substrates of the catalytic domain.

Again, while not wishing to be bound to any particular theory, for certain adzymes it is expected that a relatively fast k_{on} rate for the targeted substrate will be desirable. A k_{on} of at least 10^3 $M^{-1}s^{-1}$ may be desirable. Other kinetic and performace parameters that may be useful in certain embodiments are described below.

In most embodiments, the modular components of an adzyme are heterologous with respect to each other, meaning that these domains are not found naturally as part of a single molecule or assembly of molecules, and accordingly, adzymes of these embodiments are not naturally occurring substances. Each of the various domains and moieties that are present in an adzyme may themselves be a naturally occurring protein or protein fragment, or other naturally occurring biomolecule (*e.g.*, a sugar, lipid or non-proteinaceous factor), or an engineered or wholly synthetic molecule.

In most embodiments, a catalytic domain will comprise a polypeptide having enzymatic activity. In certain preferred embodiments, a targeting moiety will comprise a polypeptide. In general, at least one catalytic domain and at least one targeting moiety of the adzyme are selected from amongst "modular" entities, i.e., able to function as a catalyst or binding agent independently. To exemplify, an adzyme may be a single fusion protein comprising (1) a catalytic domain that comprises a polypeptide and has enzymatic activity and (2) an targeting domain that comprises a polypeptide and binds to an address binding site, and, optionally, (3) a polypeptide linker configured such that the catalytic domain and targeting domain are operatively associated. As another example, an adzyme may be a type of immunoglobulin fusion construct, wherein a first fusion protein comprises a catalytic domain fused to a first Fc chain and a second fusion protein comprises a targeting domain fused to a second Fc chain, and wherein the first and second Fc chains are associated in such a way as to cause the catalytic domain and the targeting domain to be operatively associated.

Within the broad category of adzymes, various subcategories or classes of adzymes may be identified. As noted above, two such classes are termed herein "direct" adzymes and "proximity" adzymes. In a direct adzyme the targeting moiety binds to a targeted substrate. The catalytic domain acts on the same type of molecule as the targeting moiety has bound. In certain embodiments, this will require the targeting moiety to dissociate from the targeted substrate in order for the catalytic domain to alter that molecule. Depending on a variety of conditions, such as the concentration of the direct adzyme and the concentration of the targeted substrate, the catalytic domain of a direct adzyme may primarily act on the targeted substrate that is or was bound by the targeting moiety, or the direct adzyme may act on one substrate while the targeting moiety is bound

to another. While not wishing to be bound to mechanism, it is generally expected that when the targeted substrate is present in relatively low concentrations (as is the case for most extracellular signaling molecules in the extracellular fluids of a multicellular organism), a direct adzyme will primarily act on the targeted substrate that is or was bound by the targeting moiety. In a proximity adzyme, the targeting moiety binds to a molecule that is not covalently part of the targeted substrate. Instead, the targeting moiety binds to a molecule that is expected to be found in functional proximity to the targeted substrate. By "functional proximity" is meant that the address binding site is present at sufficient concentration or with sufficient stability in the proximity of targeted substrates that the adzyme reacts with the targeted substrate with greater effectiveness than the catalytic domain and targeting moiety alone or in non-associated combination. While the existence of functional proximity between an address binding site and a targeted substrate is most accurately assessed in the milieu in which the adzyme is intended for use (*e.g.*, in the human body, in a contaminated soil site), an adzyme may be considered a proximity adzyme if it shows the appropriate effectiveness in a reasonable experimental system, such as a culture of cells related to the type of cells that are predicted to be targeted by the adzyme, or in a purified protein mixture where the address binding site and the adzyme are present at concentrations that fairly approximate those that are expected in the intended milieu. In certain embodiments, the targeting moiety binds to a molecule which is diffusionally constrained with respect to the targeted substrate, meaning that, for whatever reason, the targeted substrate and the address binding site are neither covalently attached nor free to diffuse apart. For example, the targeting moiety may bind one protein in a receptor complex while the catalytic domain acts on another protein in the receptor complex. As another example, the targeting moiety may bind to a protein that is lodged in cell membranes and the targeted substrate may also be lodged in or attached to cell membranes. The terms "direct adzyme" and "indirect adzyme", while distinct concepts that raise different issues in adzyme design, may not, in practice, be entirely mutually exclusive. For example, an targeting moiety may bind to both the targeted substrate and a separate molecule that occurs in functional proximity to the targeted substrate.

An additional discernible class of adzymes are the "contingent adzymes". The term "contingent adzymes" refers to adzyme constructs that are catalytically activated or up-regulated in the vicinity of the targeted substrate but less active, such as by inhibition, elsewhere. Both direct and proximity adzymes can be modified to be contingent adzymes, in which the interaction of the targeting domain with its cognate partner alters the activity of the catalytic domain, such as by allosteric, competitive, or non-competitive mechanisms.

As a descriptive example, a variety of antibodies with affinity for particular targets (e.g., anti-TNF-α and anti-EGF receptor) have been used as effective therapeutic agents for certain disorders, and it is expected, in accordance with the teachings herein, that adzymes with greater potency than the antibodies alone may be designed.

In a further aspect, the present invention provides pharmaceutical compositions comprising an adzyme of the invention and a pharmaceutically acceptable carrier, as well as methods for making a medicament for use in a human by combining an adzyme with a pharmaceutically acceptable carrier.

In another aspect, the present invention provides a method for treating a subject, *e.g.*, a human, suffering from a disease. The method includes administering (*e.g.*, using a pharmaceutical formulation) a therapeutically, prophylactically or analgesically effective amount of an adzyme, thereby treating a subject suffering from a disease. In one embodiment, the disease is associated with a soluble molecule and the adzyme is administered to the subject in an amount effective to render the soluble molecule biologically inactive.

II. Definitions

For convenience, certain terms employed in the specification, examples, and appended claims are collected here. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein, the term "aptamer", when referring to a targeting moiety, encompasses an oligonucleotide that interacts with a targeted substrate or associated molecule, e.g., binds to the address site for an adzyme.

As used herein, the term "biologically inactive" as it relates to a targeted biomolecule is intended to mean that its biological function is down-regulated, e.g., suppressed or eliminated. For example, if the target is TNFα, biological inactivation would include modifying TNFα such that the inflammatory response mediated by NFKB is inhibited, there is inhibition of the secretion of other pro-inflammatory cytokines, the induction of endothelial procoagulant activity of the TNF is inhibited; the binding of TNF to receptors on endothelial cells is inhibited; the induction of fibrin deposition in the tumor and tumor regression activities of the TNF are enhanced; and/or the cytotoxicity and receptor binding activities of the TNF are unaffected or enhanced on tumor cells. For example, a catalytic domain capable of methylating TNFα (*e.g.*, methylating TNFα on ^{15}His as described in Yamamoto R. *et al.* (1989) *Protein Engineering* 2(7):553-8) would inactivate TNFα.

The term "k_{cat}", or the "turnover number", is the number of substrates converted to product per enzyme molecule per unit of time, when E is saturated with substrate.

The term "k_{cat}/K_m", is an apparent second-order rate constant that is a measure of how the enzyme performs when the concentration of substrate is low (e.g., not saturating). The upper limit for k_{cat}/K_m is the diffusion limit – i.e., the rate at which enzyme and substrate diffuse together. k_{cat} / K_m is also known as the "catalytic efficiency" for the enzyme.

The term "catalytic efficiency", as applied to an adzyme, is the apparent second-order rate constant of the adzyme when the concentration of substrate is substantially (at least ten-fold) lower than the Michaelis-Menten constant (K_m) for the adzyme (*i.e.*, when $[S] << K_m$), at least with respect to those adzymes that can be reasonably modeled using Michaelis-Menten kinetic modeling theories. In the case of many simple catalytic domains taken in isolation, the catalytic efficiency may be defined as the ratio k_{cat}/K_m (see above).

In most cases where Michaelis-Menten modeling applies, the catalytic efficiency will be different for the adzyme and for its component enzyme, i.e. the adzyme's catalytic efficiency is not k_{cat} / K_m. Both v_{max} and K_m are also different for the adzyme. For a case where the Michaelis-Menten pseudo-steady state analysis is valid (generally $[AE]_o << [S]_o$, wherein $[AE]_o$ is the initial adzyme concentration) and substrate holdup is negligible, simple closed-form expressions for these quantities can be derived:

$$v_{\max}^{AE} = \frac{k_{off}^{AS}}{k_{cat}^{ES} + k_{off}^{AS} K_m^E / [S]_{eff} + k_{off}^{AS}} v_{\max}^{E}$$

$$K_m^{AE} = \frac{(k_{off}^{AS} K_m^E / [S]_{eff} + k_{cat}^{ES}) k_{off}^{AS}}{(k_{cat}^{ES} + k_{off}^{AS} K_m^E / [S]_{eff} + k_{off}^{AS}) k_{on}^{AS}}$$

wherein v_{max}^{AE} and v_{max}^{E} are the maximum velocity for the adzyme and its enzyme component, respectively; K_m^{AE} and K_m^{E} are the K_m for the adzyme and its enzyme component, respectively. The superscript "AS" indicates that the kinetic constant is that of an address / targeting moiety, which is determined by independent experiments on the address; the superscript "ES" indicates that the kinetic constant is that of an enzyme / catalytic moiety, which is determined by independent experiments on the enzyme. $[S]_{eff}$ or the "effective concentration" of the targeted substrate is a geometric parameter of the adzyme with concentration units.

The catalytic efficiency for an adzyme is:

$$\text{Catalytic Efficiency} = \frac{v_{\max}^{AE}}{K_m^{AE}[AE]_o} = \frac{k_{on}^{AS} k_{cat}^{ES}}{k_{off}^{AS} K_m^E / [S]_{eff} + k_{cat}^{ES}}$$

A "chimeric protein construct" is an assemblage comprising at least two heterologous moieties, e.g., a catalytic domain and an address that are heterologous with respect to each other, that are covalently or non-covalently associated to form a complex. A chimeric protein construct may comprise non-proteinaceous molecules.

"Differentiation" in the present context means the formation of cells expressing markers known to be associated with cells with different functional properties or cells that are more specialized and closer to becoming terminally differentiated cells incapable of further division or differentiation.

A "fusion protein" is a chimeric protein wherein at least two heterologous amino acid sequences are covalently joined through an amide backbone bond, e.g., to form one contiguous polypeptide.

As used herein, the terms "modulate" or "alter" the activity of the targeted substrate are intended to include inhibiting, stimulating, up-regulating, down-regulating, activating, inactivating, or modifying the activity of the target in any other way.

A polynucleotide sequence (DNA, RNA) is "operatively linked" to an expression control sequence when the expression control sequence controls and regulates the transcription and translation of that polynucleotide sequence. The term "operatively linked" includes having an appropriate start signal (e.g., ATG) in front of the polynucleotide sequence to be expressed, and maintaining the correct reading frame to permit expression of the polynucleotide sequence under the control of the expression control sequence, and production of the desired polypeptide encoded by the polynucleotide sequence.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention, i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

The terms "polynucleotide sequence" and "nucleotide sequence" are also used interchangeably herein.

As used herein, "protein" is a polymer consisting essentially of any of the 20 amino acids. Accordingly, a protein may include various modifications (e.g., glycosylation, phosphorylation) or non-amino acids. Although "polypeptide" is often used in reference to relatively large polypeptides, and "peptide" is often used in reference to small polypeptides, usage of these terms in the art overlaps and is varied.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

The International Union of Biochemistry and Molecular Biology (1984) has recommended to use the term "peptidase" for the subset of peptide bond hydrolases (Subclass E.C 3.4.). The widely used term protease is synonymous with peptidase. Peptidases comprise two groups of enzymes: the endopeptidases and the exopeptidases. Endopeptidases cleave peptide bonds at points within a protein, and exopeptidases remove amino acids sequentially from either the N- or C-terminus.

The term "proteinase" is also used as a synonym for endopeptidase. Proteinases are classified according to their catalytic mechanisms. Five mechanistic classes have been recognized by the International Union of Biochemistry and Molecular Biology: serine proteinases, cysteine proteinases, aspartic proteinases, threonine proteinases, and metalloproteinases.

This classification by catalytic types has been suggested to be extended by a classification by families based on the evolutionary relationships of proteases (Rawlings, N.D. and Barrett, A.J., (1993), Biochem. J., 290, 205-218). This classification is available in the SwissProt database.

In addition to these five mechanistic classes, there is a section of the enzyme nomenclature which is allocated for proteases of unidentified catalytic mechanism. This indicates that the catalytic mechanism has not been identified, and the possibility remains that novel types of proteases do exist.

The class "serine proteinases" comprises two distinct families: the chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein, and the substilisin family which includes the bacterial enzymes such as subtilisin. The general three-dimensional structure is different in the two families but they have the same active site geometry and catalysis proceeds via the same mechanism. The serine proteinases exhibit different substrate specificities which are related to amino acid substitutions in the various enzyme subsites (see the nomenclature of Schechter and Berger) interacting with the substrate residues. Three residues which form the catalytic triad are essential in the catalytic process: His-57, Asp-102 and Ser-195 (chymotrypsinogen numbering).

The family of "cysteine proteinases" includes the plant proteases such as papain, actinidin or bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated), and several parasitic proteases (e.g., Trypanosoma, Schistosoma). Papain is the archetype and the best studied member of the family. Like the serine proteinases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys-25 and His-159 (papain numbering) play the same role as Ser-195 and His-57 respectively. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His-159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps and then a water molecule is not required.

Most of the "aspartic proteinases" belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D, processing enzymes such as renin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin). A second family comprises viral proteinases such as the protease from the AIDS virus (HIV) also called retropepsin. In contrast to serine and cysteine proteinases, catalysis by aspartic proteinases does not involve a covalent intermediate, though a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfers: one from a water molecule to the dyad of the two carboxyl groups and a second one from the dyad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. This general acid-base catalysis, which may be called a "push-pull" mechanism leads to the formation of a non-covalent neutral tetrahedral intermediate.

The "metalloproteinases" are found in bacteria, fungi as well as in higher organisms. They differ widely in their sequences and their structures but the great majority of enzymes contain a zinc (Zn) atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity. Bacterial thermolysin has been well characterized and its crystallographic structure indicates that zinc is bound by two histidines and one glutamic acid. Many enzymes contain the sequence HEXXH (SEQ ID NO:), which provides two histidine ligands for the zinc whereas the third ligand is either a glutamic acid (thermolysin, neprilysin, alanyl aminopeptidase) or a histidine (astacin). Other families exhibit a distinct mode of binding of the Zn atom. The catalytic mechanism leads to the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group.

In discussing the interactions of peptides with proteinases, e.g., serine and cysteine proteinases and the like, the present application utilizes the nomenclature of Schechter and Berger [(1967) Biochem. Biophys. Res. Commun. 27:157-162)]. The individual amino acid residues of a substrate or inhibitor are designated P1, P2, etc. and the corresponding subsites of the enzyme are designated S1, S2, etc. The scissile bond of the substrate is P1-P1'.

The binding site for a peptide substrate consists of a series of "specificity subsites" across the surface of the enzyme. The term "specificity subsite" refers to a pocket or other site on the enzyme capable of interacting with a portion of a substrate for the enzyme.

"Recombinant," as used herein with respect to a protein, means that the protein is derived from the expression of a recombinant nucleic acid by, for example, a prokaryotic, eukaryotic or in vitro expression system. A recombinant nucleic acid is any non-naturally occurring nucleic acid sequence or combination of nucleic acid sequences that was generated as a result of human intervention.

The term "substrate" refers to a substrate of an enzyme which is catalytically acted on and chemically converted by the enzyme to product(s).

The term "stereoisomers" refers to compounds which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space. In particular, "enantiomers" refer to two stereoisomers of a compound which are non-superimposable mirror images of one another. "Diastereomers", on the other hand, refers to stereoisomers with two or more centers of asymmetry and whose molecules are not mirror images of one another. With respect to the nomenclature of a chiral center, terms "D" and "L" configuration are as defined by the IUPAC Recommendations. As to the use of the terms, diastereomer, racemate, and enantiomer will be used in their normal context to describe the stereochemistry of peptide preparations.

"Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to nucleic acid sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In some examples, transcription of a recombinant gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of a protein.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. Preferred vectors are those

capable of autonomous replication and/or expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors".

III. Exemplary Embodiments

An adzyme comprises at least two modular moieties: a targeting moiety and a catalytic domain. With respect to altering the activity of a targeted substrate, the adzyme is more potent relative to either the catalytic domain or targeting moiety alone.

The catalytic domain will often be protein-based, though even then may include other components, such as organic ligands or co-factors, or metal ions. It comprises a catalytically active site that reacts with a substrate without itself being consumed in the reaction. A catalytic domain will generally alter one or more bonds of a substrate, e.g., breaking the bond, removing one or more atoms across the bond (including oxidizing or reducing), and/or altering the stereochemistry of an atom participating in the bond. The site of chemical modification on the targeted substrate is referred to herein as the "substrate site".

The targeting moiety recognizes and binds to a pre-determined molecule, *i.e.*, an address binding site such as on a soluble or membrane bound intracellular or extracellular targeted biomolecule, which molecule is the same as or associated with the targeted substrate. The effect in both instances is to impart "addressability" to the adzyme construct, that is, to increase the local concentration of the construct in the vicinity of the targeted substrate so as to increase the proximity of the catalytic domain to the targeted substrate and thereby increase the catalytic efficiency for that substrate.

The targeting moiety and catalytic domain may be covalently attached or associated by non-covalent means. For instance, the moieties can be covalently attached as by fusion of two protein domains, with or without intervening sequences, to form a single polypeptide chain, or through derivation of the amino or carboxy terminus, or a sidechain of a polypeptide chain. In certain preferred embodiments, the targeting moiety and catalytic domain are produced as a cotranslational fusion by expression of a single recombinant nucleic acid construct. The various moieties may also be associated by non-covalent interactions, such as between protein domains, interaction with a common cross-linking ligand, etc.

The adzyme concept can be exploited in appropriate circumstances using a recruitment approach. Here, a multispecific binder is administered. An address of the multispecific binder complexes with a binding site on or near the intended targeted biomolecule. A chaperone protein or other structure of the multispecific binder, linked to

or constituting a part of the address, displays a surface which complexes with a catalytic domain such as an enzyme already present in the body, or a co-administered enzymatically active moiety. The multispecific binder thereby induces complex formation between the address and a catalytic domain. The affinity of the address for the binding site serves to increase the effective concentration of the catalytic domain in the vicinity of the targeted biomolecule.

The address and catalytic domain of an adzyme often cooperate to produce synergistic behavior. The target may be modulated, e.g., inhibited by cleavage, by a catalytic domain used alone at a potency determined by its K_m and k_{cat}. The target may also be inhibited by binding with a molecule defining an address used alone at a potency determined by its K_a, acting simply as a conventional drug. The amount of modulation of the target often may be objectively measured by standard assays. Thus modulation induced independently through each mechanism often can be at least roughly quantitated. It often will be found, at least in some adzyme constructs, that an adzyme comprising an optimized combination of a catalytic domain having the same K_m and k_{cat}, and an address having the same K_a will have a potency at least 10, 10^2, 10^3, or even 10^4 times the sum of the potency of the individual components (catalytic and targeting) acting alone.

Another way to express the functional improvement of the adzyme in a pharmaceutical setting, relative to the targeting moiety and/or catalytic domain alone, is that in certain preferred embodiments the adzyme will have an effective dose (ED_{50}) for altering the activity of the targeted substrate in vivo at least 2 times less than the catalytic domain and/or targeting moiety (e.g., if a neutralizing moiety) alone, and more preferably at least 5, 10 or even 100 times less.

In the case of embodiments in which the targeted substrate is degraded to an inactive form by the adzyme, the potency may be expressed in terms of "HL_{50}", e.g., the concentration of adzyme required to reduce the half-life (T½) in vivo of the targeted substrate by 50 percent. The more potent and selective the adzyme is, the lower the HL_{50} concentration is relative to the catalytic domain alone. In certain preferred embodiments, the HL_{50} of the adzyme is at least 2 times less than the catalytic domain alone, and more preferably at least 5, 10 or even 100 times less.

In certain embodiments, the adzyme has a catalytic efficiency for the catalyzed reaction with the targeted substrate of at least 10^4 $M^{-1}sec^{-1}$, and even more preferably at least 10^5 $M^{-1}sec^{-1}$ or even at least 10^6 $M^{-1}sec^{-1}$.

In certain embodiments, the adzyme has a catalytic efficiency for the catalyzed reaction with the targeted substrate at least 5 times greater than the catalytic domain alone, and even more preferably at least 10, 50 or even 100 times greater.

In certain therapeutic applications, it will be important to balance the potency and specificity of an adzyme. A good balance of potency and specificity can be achieved through the following design criterion:

$$k_{cat}^{ES} / K_m^E = k_{off}^{AS} / [S]_{eff}$$

In adzyme embodiments designed with this criterion, the catalytic domain will be very weak, in some cases having a catalytic efficiency as low as 100, 10, or 1 $M^{-1}s^{-1}$, or even lower. Thus, adzymes designed to balance potency and specificity should be derived from weak enzyme domains.

In certain embodiments, the k_{off} rate of the targeting moiety will be similar for the substrate and the adzyme reaction product, and it will be desirable to optimize the k_{off} rate for high substrate affinity and rapid release of the product when bound to the address. In these embodiments, the optimal k_{off} rate may be 0.001 sec^{-1}, 0.01 sec^{-1}, 0.1 sec^{-1}, or greater, and can be approximated by:

$$k_{off,optimal}^{AS} \approx \sqrt{k_{on}^{AS} \frac{k_{cat}}{K_m^E} [S]_{eff} [S]}$$

when $[S]_{eff} << K_m^E$, wherein K_m^E is the enzyme's K_m (not the adzyme's). The k_{on}^{AS} (k_{on} of adzyme) above is the same as k_1 in Equation 2 below.

For a fusion protein of two domains both of which independently bind the substrate, the "effective concentration of a substrate," $[S]_{eff}$, is the quotient of the overall association equilibrium constant for the fusion protein binding to its substrate and the product of the association equilibrium constants for the two, independent address domains binding to the substrate. This definition follows Figure 1 and Equation 2 in Zhou, *J. Mol. Biol.* (2003) **329**, 1-8. Each of the three equilibrium constants required to determine $[S]_{eff}$ can be measured via standard binding assays. In performing kinetic analysis, it is further assumed that the microscopic off rates for each domain in a fusion protein are not affected by the presence of the linker.

In certain embodiments, the adzyme has a K_m for catalyzed reaction with the targeted substrate at least 5 times less than the catalytic domain alone, and even more preferably at least 10, 50 or even 100 times less.

Broadly, the adzyme may be designed to interact with any biomolecule target provided the site of enzymatic attack and the binding site for the address are solvent accessible. Thus, both the targeted biomolecule and the binder for the address may be a soluble biomolecule or a membrane-bound biomolecule. The target may be intracellular,

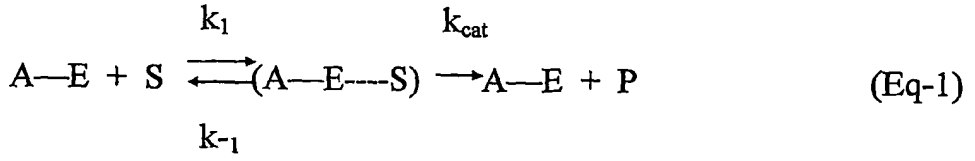
although extracellular targets are more accessible to protein constructs and are therefore preferred.

Referring to Figure 1, schematic diagrams illustrative of various structures which can exploit the invention are set forth as Figs. 1A through 1K. In 1A, perhaps the simplest adzyme, an address (ADD) is covalently linked to a catalytic domain (CAT). Such a construct may be embodied as two separate globular protein domains attached by a flexible or rigid linker as illustrated,, or by a single globular protein wherein one portion of the molecular surface functions as the address and another as a catalytically active site. In Figure 1B, the domains are complexed, i.e., each comprises a surface that reversibly binds to a surface on its partner. In Figures 1C through 1F, the address and catalytic domains are associated via a chaperone protein, with either or both linked to the chaperone via covalent bonds such as a linker or noncovalent protein-protein complexation. In Figures 1G and 1H, each of the address and catalytic domains is linked, covalently or non covalently, to a chaperone protein domain, and the chaperone domains are noncovalently complexed together.

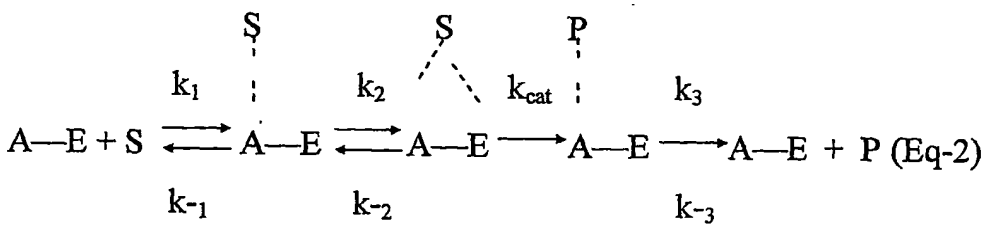
Figures 1I and 1J illustrate one way to exploit the recruitment embodiment of the invention. These constructs, as illustrated, comprises an address linked (covalently or non covalently) to a chaperone protein, which defines a binding surface specific for a predetermined catalytic domain, i.e., an enzyme either already present in a body fluid or one co administered with the construct. This type of construct functions by recruiting the enzyme to the vicinity of the targeted biomolecule, mediated by the affinity of the address for the target so that the fully functional adzyme is assembled *in vivo*. Of course, such enzyme recruiting constructs could also be embodied in other forms provided they have a binding surface serving as an address that binds to the binding site on or adjacent the target, and a binding surface that serves to bind specifically to an enzyme. For example, a recruitment construct may be embodied as a single globular protein, or as a globular protein defining a binding surface for a catalytic domain and a small molecule with affinity for the target linked to it through a length of biocompatible polymer.

After the enzymatic reaction is complete, the adzyme disassociates from the target (now converted to a product) and moves on to bind to and act on another molecule of the target, creating turnover. As a result of this feature of the adzymes, the potency of the drug constructs is not dependant directly on drug/target stoichiometry. This provides a significant engineering advantage and can permit avoidance of toxicity issues associated with the use of antibodies or small molecule drugs inhibiting soluble biomolecules associated with a disease.

The equations below illustrates two possible adzyme (A—E) interactions between an address (A) and its binding site on a targeted biomolecule (S), and between the adzyme's enzymatically active site (E) and the targeted substrate (S) to make product (P).



$$A—E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (A—E\text{----}S) \xrightarrow{k_{cat}} A—E + P \qquad \text{(Eq-1)}$$



$$A—E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A—E \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} A—E \xrightarrow{k_{cat}} A—E \xrightarrow{k_3} A—E + P \qquad \text{(Eq-2)}$$

Reaction 1 is the normal catalytic reaction, where the address is not involved, such as might occur with a substrate that does not display a binding site for the address. In the presence of a local concentration of both the adzyme (A—E) and the biomolecule (S) the targeted substrate has an on rate k_1 for the enzyme pocket (E), forms a complex A—E---S with the pocket, and is converted at a rate dependent on k_{cat} to product P and released.

Reaction 2 occurs when the binding site on the targeted substrate S binds to the adzyme through formation of an address: binding site interaction (with an affinity that may be higher than the E---S affinity), forming a complex S---AE with on rate k_1. Presuming a suitable structure of the adzyme, e.g., the length of the linker or stereochemistry of the complex and its target permits, this complex can enter an intermediate state at rate k_2 where the targeted substrate interacts simultaneously with the address and the enzyme pocket. In this state the targeted substrate is converted to product P at a rate governed by k_{cat}, and then disassociates from the adzyme at rate k_3.

The functioning and structure of various forms of adzymes may be understood better with reference to Figures 2A-2J. *Figure 2A* depicts an adzyme in situ at a moment when it has bound to its intended biomolecule. In this case the adzyme is embodied as a single globular protein which defines a catalytic domain (CD) having an enzymatically active site and an address (AD) defined by a separate surface on the protein. The address binds reversibly with a binding site, in this case embodied as a surface on the targeted

biomolecule. The targeted substrate site is vulnerable to immediate enzymatic attack by the enzymatically active site of the catalytic domain.

Figure 2B shows a construct similar to Figure 2A except that the address is a small molecule attached to the catalytic domain by a flexible linker that binds reversibly directly with a binding site on the intended targeted biomolecule.

Figure 2C is an adzyme similar to 2B in which the address and the catalytic domain are attached by a flexible leash. Binding of the address domain to the binding site, here again illustrated as a portion of the targeted biomolecule, serves effectively to increase the local concentration of the catalytic domain in the region of the target, as illustrated. The address domain and the catalytic domain may be linked via a flexible linker, or a more rigid structure (not shown) such that binding of the address domain serves to pose the catalytic domain in position to induce chemical change in its targeted biomolecule.

The adzyme of *Figure 2D* is similar to Figure 2C, except that the binding site and the targeted biomolecule are separate molecular species, here illustrated as being lodged in a membrane, such as a cell membrane. As in the embodiments of Figures 2A-2C, binding of the address domain to the recognition site of what here functions as a attractant molecule serves to effectively increase the local concentration of the catalytic domain in the region of the target. Where the concentration of two proteins on a cell is significant, especially in cases where they are known to interact in lipid rafts or the like, one molecule can be used as the binding site to attract the construct to the other molecule that will be catalytically modulated.

The adzyme of *Figure 2E* is similar to Figure 2C, except that the address domain and the catalytic domain are non-covalently associated directly to each other. Examples of this type of association include dimerization, optionally stabilized by disulfide linkages, hybridization of complementary nucleotides, or protein-protein complexation of the type that is ubiquitous within cells.

Figure 2F shows an embodiment of an adzyme similar to Figure 2E, except that the address domain is designed to bind to an attractant biomolecule separate from but complexed to the targeted biomolecule. Nevertheless, binding increases the effective concentration of the target and its substrate site in the vicinity of the catalytic domain as shown.

Figure 2G is the same as Figure 2F except that the targeted biomolecule is complexed with a separate protein displaying the binding site through a third, complexing protein.

Figure 2H illustrates an embodiment of an adzyme in which the address and the catalytic domain are non-covalently associated through a third, chaperone protein, to form an active complex. Its intended targeted biomolecule is illustrated as being embedded in a lipid bilayer, and the binding site is illustrated as residing on a separate molecule in the lipid bilayer, similar to Figure 2D. Again, binding nevertheless increases the effective concentration of the target and its substrate site in the vicinity of the catalytic domain.

Figure 2I illustrates an embodiment of an adzyme similar to Figure 2H, except that the address domain binds to a binding site directly on the targeted biomolecule.

Figure 2J is similar to Figure 2G, except that the address domain and catalytic domain of the adzyme are held together via complexation with a chaperone protein. In all construct where the AD and CD are non covalently complexed, the surface on the address domain that binds to the catalytic domain (or a chaperone protein) may be the same or different from the one that binds to the binding site on the target or trigger molecule.

A further optional feature of adzymes is "engineered contingency," that is, creation of a family of adzymes that *become* capable of reacting with their target in the presence of the target or another triggering or attractant molecule having an affinity for the address. Figure 1K illustrates the fundamental idea behind the contingent adzyme. As illustrated, the address has an affinity for the catalytic domain and is configured so that it can bind to it and inhibit its enzymatic activity. In the presence of the target, a competition for the address ensues, freeing the catalytic domain to induce chemical change in its intended target.

Stated differently, contingent adzyme constructs are inactive (have low enzymatic activity) in the absence of a triggering molecule, but become active in the presence of the triggering molecule, e.g., the target (see Legendre D. *et al.* (1999) *Nature Biotechnology* 17:67-72; Legendre D. *et al.* (2002) *Protein Science* 11:1506-1518; Soumillion P. and Fastrez J. (2001) *Current Opinion in Biotechnology* 12:387-394). This type of adzyme also requires a catalytic domain and an address. However, in this case, binding of the address has the effect of freeing up the catalytic site of the catalytic domain to enhance its activity. This may be achieved in several ways, illustrated by way of example in Figures 3A through 3G, which are described in more details in the contingent adzyme section.

In addition to the address and catalytic domains, and the optional chaperone proteins, linkers and other structures defining the relationship of these parts, an adzyme may further comprise one or more fusion partners operatively linked to any of its components, e.g., N- terminal or C-terminal fusions, or added or substituted sequences in loops on protein domains. Adzymes may also include polymeric side chains, small molecules, or metal ions. These moieties may, for example, restrict the adzyme to a

conformationally restricted or stable form; serve as a targeting sequence allowing the localization of the adzyme into a sub-cellular or extracellular compartment; assist in the purification or isolation of either the adzyme or the nucleic acids encoding it; serve to confer a desired solubility on the adzyme; or confer stability or protection from degradation to the adzyme or the nucleic acid molecule(s) encoding it (*e.g.*, resistance to proteolytic degradation). The adzyme may comprise one or any combination of the above fusion partners as needed.

The fusion partners can, for example, be $(histidine)_6$-tag, glutathione S-transferase, protein A, dihydrofolate reductase, Tag•100 epitope (EETARFQPGYRS; SEQ ID NO:1), c-myc epitope (EQKLISEEDL; SEQ ID NO:2), FLAG®-epitope (DYKDDDK; SEQ ID NO:3), lacZ, CMP (calmodulin-binding peptide), HA epitope (YPYDVPDYA; SEQ ID NO:4), protein C epitope (EDQVDPRLIDGK; SEQ ID NO:5) or VSV epitope (YTDIEMNRLGK; SEQ ID NO:6).

The fusion partner may also be a membrane translocation domain, *i.e.*, a peptide capable of permeating the membrane of a cell and which is used to transport attached peptides into or out of a cell *in vivo*. Membrane translocation domains that may be used include, but are not limited to, the third helix of the *antennapedia* homeodomain protein and the HIV-1 protein Tat or variants thereof. Additional membrane translocation domains are known in the art and include those described in, for example, Derossi *et al.*, (1994) *J. Biol. Chem.* 269, 10444-10450; Lindgren *et al.*, (2000) *Trends Pharmacol. Sci.* 21, 99-103; Ho *et al.*, *Cancer Research* 61, 474-477 (2001); U.S. Patent No. 5,888,762; U.S. Patent No. 6,015,787; U.S. Patent No. 5,846,743; U.S. Patent No. 5,747,641; U.S. Patent No. 5,804,604; and Published PCT applications WO 98/52614, WO 00/29427 and WO 99/29721.

A. Exemplary Targeting Moieties

It will be appreciated that a wide range of entities can be used as targeting moieties in the subject adzymes. Fundamentally, the targeting moiety reversibly binds to a pre-determined feature ("address site") associated with the targeted substrate. The targeting moiety presents one or more surfaces having chemical characteristics (e.g., hydrophobic, steric and/or ionic) which permit it to bind selectively, or relatively selectively, with the address site. In many embodiments, the address will be a modular protein (including peptide) domain which is provided in association with the catalytic domain. For example, the targeting moiety can be an antibody, or a fragment of an antibody which retains the ability to bind to the address site. Accordingly, the targeting moiety can be derived from such antibody and antibody fragments as monoclonal antibodies, including Fab and F(ab)2

fragments, single chain antibodies (scFv), diabodies, and even fragments including the variable regions of an antibody heavy of light chain that binds to the address site.

Other examples of proteins that can be suitably adapted for use in the subject adzymes including ligand binding domains of receptors, such as where the targeted substrate of the adzyme is the receptor ligand. Conversely, the targeting moiety can be a receptor ligand where the adzyme is directed to the receptor as the targeted substrate. Such ligands include both polypeptide moieties and small molecule ligands.

In still other embodiments, the targeting moiety can be an engineered polypeptide sequence that was selected, e.g., synthetically evolved, based on its kinetics and selectivity for binding to the address site.

The targeting moiety can also be a polyanionic or polycatonic binding agent, such as an oligonucleotide, a polysaccharide, a polyamino peptide (such as poly-aspartate, poly-glutamate, poly-lysine or poly-arginine). In certain embodiments, such targeting moieties maintain a number of either negative or positive charges over their structure at physiological pH. The address may also be a protein nucleic acid (PNA), a lock nucleic acid (LNA) or a nucleotide sequence, such as a single strand of DNA or RNA.

The targeting moiety may also be a small molecule that has been selected based on the kinetics and selectivity it displays for binding to an address site associated with the targeted substrate.

There are a variety of well-known techniques for generating libraries of polypeptide/peptide, nucleic acid (aptamer) and small molecule moieties that can be used to identify molecules having the appropriate specificity, selectivity and binding kinetics for use in any particular adzyme. For example, such techniques as described in US Patents 6258558 titled "Method for selection of proteins using RNA-protein fusions" and 5837500 titled "Directed evolution of novel binding proteins" can be readily adapted for use in identifying peptide or polypeptide targeting moieties for use in generating the subject adzymes. Likewise, the preparation of aptamers previously described in the art can be adapted for generating appropriate targeting moieties. See, for example, Tuerk Science 249:505-510 (1990); Klug Mol Biol Reports 20:97-107 (1994); and Morris et al, PNAS 95:2902-2907 (1998), as well as U.S. Patents 5,843,701 and 5,843,653.

The address may be at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90 or 100 amino acid residues long. Ranges using a combination of any of the foregoing recited values as upper and/or lower limits are intended to be included in the present invention.

In certain preferred embodiments, the dissociation constant (K_d) for binding to the address site is lower (higher affinity) and/or the K_{off} rate is slower when the address site is

bound to the unmodified targeted substrate relative to when it is bound to the adzyme reaction product (e.g, the targeted substrate that has been acted on by the catalytic domain). That is, conversion of the targeted substrate to an adzyme reaction product reduces the affinity of the targeting moiety for the address binding site and promotes dissociation of the adzyme from the reaction product. In certain embodiments: the K_d of the targeting moiety for the adzyme reaction product relative to the targeted substrate is at least 5 times greater, and even more preferably 10, 100 or even 1000 times greater; and/or the K_{off} rate of the targeting moiety for the adzyme reaction product is at least 5 times faster, and even more preferably 10, 100 or even 1000 times faster relative to the K_{off} rate for the targeted substrate.

In certain embodiments of direct adzymes, the address site and substrate site are overlapping in the sense that binding of the targeting moiety to the targeted substrate interferes with the ability of the catalytic domain to act on the targeted substrate site. This interference may be the result of steric occlusion, or the lack of flexility in the adzyme and/or targeted substrate to permit both portions of the adzyme to simultaneously interact with the targeted substrate. In other embodiments, the address and substrate sites are spaced sufficiently apart, and the adzyme has sufficient steric flexibility, that dissociation of the targeting moiety is not required for the adzyme to modify the targeted substrate. In many embodiments, the adzyme will be designed such that there is functional cooperativity between the catalytic domain and targeting moiety, particularly resulting from appropriate selection of linker(s) between the two components, such that the affinity of the resulting adzyme is at least 2 times greater than the sum of the affinities of the catalytic domain and targeting moiety, and even more preferably at least 5, 10, 100 or even 500 times greater.

In some instances, the targeting moiety itself interferes with the activity of the targeted substrate. For example, the targeting moiety may be a blocking or neutralizing agent that inhibits an intrinsic activity or interaction mediated by the targeted substrate. In such cases, the adzyme with preferably be at least 5 times more potent an inhibitor, and even more preferably at least 10, 100 or even 1000 times more potent than the targeting moiety alone.

In other embodiments, the targeting moiety does not itself have any significant effect on the activity of the targeted substrate.

Where there are more than one possible substrate site of the catalytic domain on a targeted substrate, such as more than one substrate recognition sequences for a proteolytic domain, the targeting moiety can be selected to enhance the selectivity/preference of the adzyme for one of the sites. This can be accomplished, for example, by using a targeting moiety that binds to the targeted substrate in a manner that sterically interferes with the

catalytic domain's ability to act at one of the sites. In other embodiments, the targeting moiety can be used to increase the concentration of the catalytic domain in the proximity of the desired substrate site.

In certain embodiments, the adzyme may include two or more address/ targeting moieties, which may be the same or different (i.e., their respective K_d may be the same or different). In such embodiments, the effective K_d of the adzyme for the targeted substrate may be as low as 10^{-15}M (femtomolar), when the effective substrate concentration $[S]_{eff}$ is greater than the highest individual K_d of the addresses (or targeting moieties).

In certain embodiments, the targeting moiety binds to a a targeted substrate which is soluble under the reaction conditions, such as a soluble protein. In many cases, these soluble protein substrates will be present in the reaction milieu at relatively low concentrations, such as less than 0.1 μM, and often at less than 10 nM. In such embodiments, and certain others herein, it may be desirable to select a targeting moiety which, when provided in the adzyme, results in a direct adzyme having a relative fast k_{on} for binding to the targeted substrate, e.g., a k_{on} of 10^3 $M^{-1}s^{-1}$ or greater, e.g., at least 10^4 $M^{-1}s^{-1}$, 10^5 $M^{-1}s^{-1}$ or even 10^6 $M^{-1}s^{-1}$.

(i) Exemplary Targeted Biomolecules

In certain embodiments, the subject adzymes are directed to biologically active molecules ("targeted biomolecule"), e.g., including solvent accessible extracellular and intracellular substrates, as well as extracellular or cytoplasmic portions of membrane associated substrates. These include, but are not limited to, substrates from among such classes as protein and peptide substrates, nucleic acids, lipids, small molecules including extracellular factors (such as steroids and neurotransmitters) and intracellular second messengers (such as phosphorylated inositol and cAMP). By modifying the functional performance of a targeted substrate of biological relevance, the subject adzymes can be used to alter such cellular processes as gene expression, morphology, cell adhesion, growth, proliferation, migration, differentiation and/or viability of cell.

Taregeted substrates can be modified by the adzyme so as to produce one or more products having one or more differences in biological activities relative to the targeted substrate (including, for example, the elimination of all or near all biological activity of the targeted substrate). For instance, for targeted substrates which are themselves enzymes, the subject adzymes can be used to alter the intrinsic enzymatic activity of those targeted substrates. To illustrate, an adzyme can used to inhibit such proteases as elastase (in the treatment of cystic fibrosis, acute respiratory distress syndrome, and emphysema) or matrix metalloproteases involved in metastasis. In other embodiments, the adzyme

alters the ability of a targeted substrate to interact with other biological moieties, e.g., such as by altering receptor-ligand interactions, protein-protein interactions, protein-lipid interactions, protein-DNA or protein-RNA interactions to name but a few. In this respect, the adzyme can be used to increase or decrease the intrinsic activity or binding activity of the targeted substrate. Adzymes can also be used to alter the half-life or biodistribution of a targeted substrate.

In certain instances, the adzyme can be used to covert a targeted substrate into a functional antagonist of the unmodified biomolecule. Merely to illustrate, in the case of a polypeptide factor that acts through a receptor interaction, rather than generate a product that is unable to interact with the cognate receptor of the targeted substrate, the adzyme can be selected so as to alter the targeted substrate to produce a product that retains the ability to bind to the receptor but not induce the level of receptor activation possible by the unmodified targeted substrate. In this way, the adzyme inhibits the function of the polypeptide factor by (a) reducing the concentration of the polypeptide factor, and (b) generating an antagonist which reduces the effective concentration of receptor for the polypeptide factor. In preferred embodiments of this system, the product has a K_i of 10μM or less for inhibiting an activity of the targeted substrate, and even more preferably has a K_i less than 10μM, 100nM, 10nM or even 1 nM.

(a) Extracellular Targets

In certain embodiments, the adzyme is directed to an extracellular target, including target molecules that are typically located entirely outside of a cell and target molecules that are inserted into a cellular membrane but have a portion that is exposed to the extracellular environment. Several categories of extracellular targets are recognizable, including, for example, diffusible extracellular molecules (*e.g.*, growth factors, serum proteins, antibodies, any diffusible small molecule, extracellular nucleotides, lipids), extracellular molecules that are part of an insoluble aggregate (*e.g.*, β-amyloid protein, constituents of atherosclerotic plaques, insoluble fibrin fibers), membrane associated proteins and other membrane bound moieties (e.g., transmembrane proteins, lipids, membrane associated polysaccharides), and constituents of or associated with an organized extracellular matrix.

Accordingly, the subject adzymes can be used to alter, e.g., inhibit or potentiate, such cell-surface mediated signaling as autocrine signaling (self-signaling), paracrine signaling (between nearby cells), and/or endocrine signaling (over a long distance, usually via the bloodstream or other bodily fluid). The subject adzymes can also be used to alter juxtacrine signaling, e.g., signaling consequences of cell contact.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/26937

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 9/00; C12P 21/06, 21/04; A61K 38/00; C07K 1/00; C07H 21/04
US CL : 435/183, 69.1, 69.7, 69.8; 530/300, 350; 536/23.2, 23.4

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/183, 69.1, 69.7, 69.8; 530/300, 350; 536/23.2, 23.4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN AND WEST.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,383,775 B1 (DUFF et al.) 07 May 2002 (07.05.2002), see the entire document.	1-155
A	~~US 6,660,492 B1 (BODE et al.) 09 December 2003 (09.12.2003), see the entire~~ document.	1-155
A,P	US 2003/0104520 A1 (ELLINGTON et al.) 05 June 2003 (05.06.2003), see the entire document.	1-155
A,P	WO 03/022858 A2 (NAJAFI-SHOUSHTARI et al.) 20 March 2003 (20.03.2003), see the abstract and the entie document.	1-155

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 February 2004 (05.02.2004)	20 MAY 2004
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Valerie Baldt-Harris for Tekchand Saidha Telephone No. 571/272-1600

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